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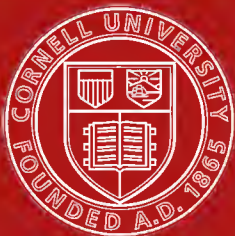
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PRACTICAL  
PHYSIOLOGICAL CHEMISTRY

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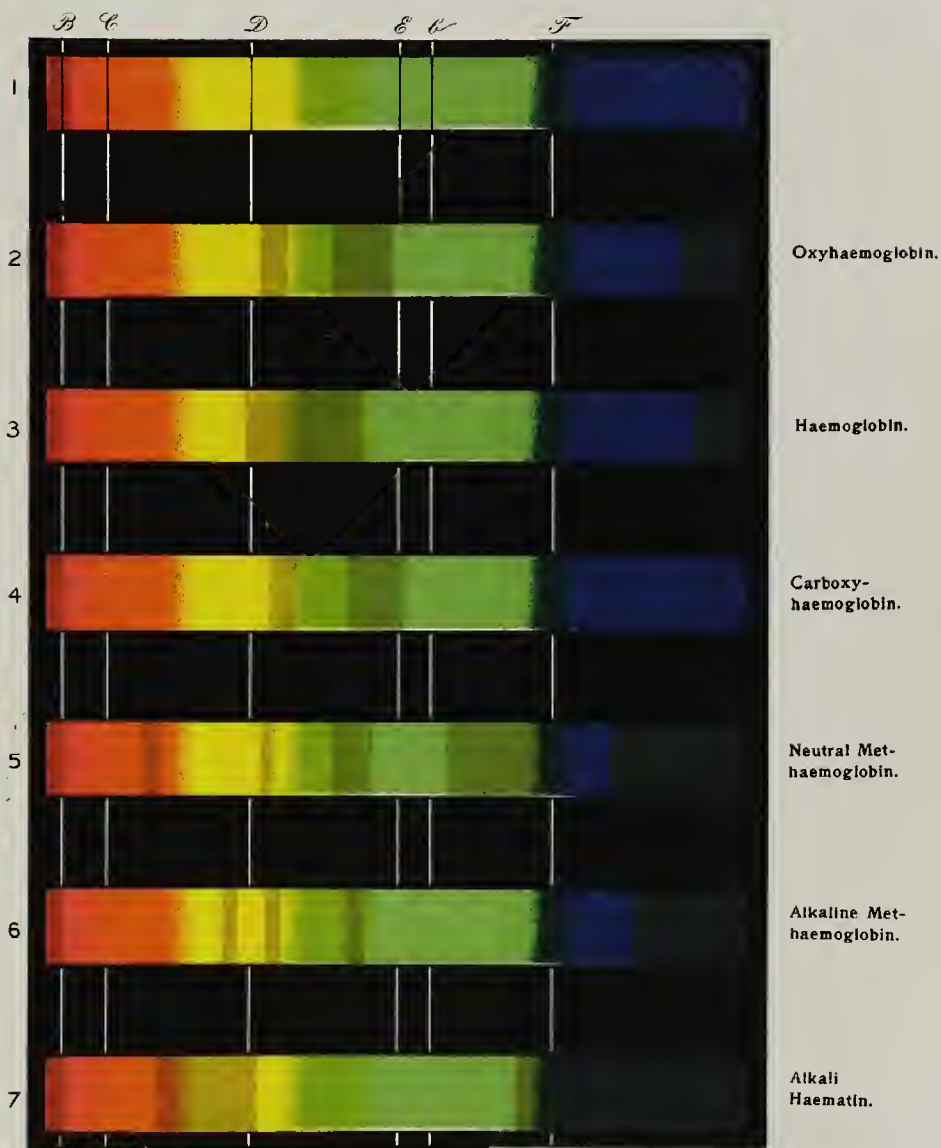
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# Absorption Spectra.

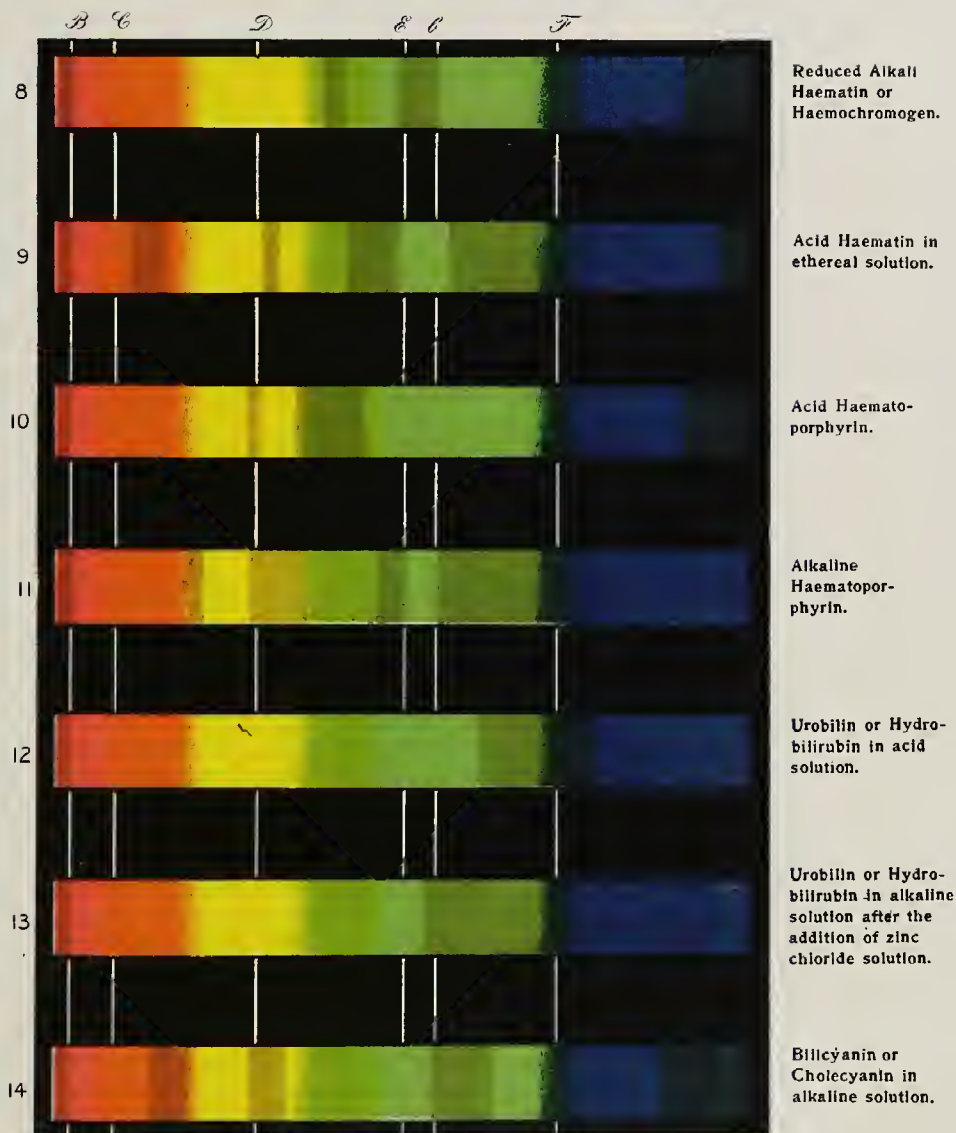
PLATE I.





# Absorption Spectra.

## PLATE II.





# PRACTICAL PHYSIOLOGICAL CHEMISTRY

A BOOK DESIGNED FOR USE IN COURSES IN PRACTICAL  
PHYSIOLOGICAL CHEMISTRY IN SCHOOLS  
OF MEDICINE AND OF SCIENCE

BY

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JEFFERSON MEDICAL COLLEGE OF PHILADELPHIA

FOURTH EDITION, REVISED AND ENLARGED

WITH TWO FULL-PAGE PLATES OF ABSORPTION SPECTRA IN COLORS  
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THESE PAGES ARE  
AFFECTIONATELY DEDICATED  
TO  
MY MOTHER



## PREFACE TO FOURTH EDITION.

The continued rapid development of the many phases of biochemistry, which has taken place in the interval of two years since the last edition of this volume appeared has necessitated a rather comprehensive revision and the consequent inclusion of considerable new matter. The main bulk of the material will be found in the chapters on Enzymes, Carbohydrates, Proteins, Blood and Lymph, Feces, Putrefaction and Quantitative Analysis of Urine. The publishers have wisely reduced the marginal space of the page thus necessitating but slight increase in the size of the volume.

The author wishes to express his gratitude to Professor William J. Gies, Professor Lafayette B. Mendel and Dr. Thomas B. Osborne for many valuable suggestions for the betterment of this revised volume. He is also under obligations to Dr. Martha Tracy and Professors Marshall P. Cram, Paul E. Howe, E. C. L. Miller, Charles J. Robinson and A. P. Sy for similar offices and to Messrs. Olaf Bergeim, Lawrence T. Fairhall, Edwin F. Hirsch, Melvin A. Saylor and Theodore F. Zucker for assistance in the verification of tests and methods, the translation of papers, the sketching of crystals and the reading of proof.

The author would be grateful if those using the volume in their classes would make suggestions regarding insertions, omissions or corrections.

PHILIP B. HAWK.

PHILADELPHIA.





## PREFACE TO THIRD EDITION.

The increasing approval with which this volume is being received has rendered necessary the preparation of a new edition, although the period elapsing since the last edition appeared is little more than one year. The present edition has been brought up to date by the insertion of various additions and corrections as well as by the inclusion of a number of qualitative tests and quantitative methods. Because of the very short intervening period since the last edition of the volume, the new material inserted is rather small in quantity when compared with that incorporated at the previous revision.

The author wishes to thank Dr. W. H. Welker and Dr. Croll for permission to insert unpublished material.

PHILIP B. HAWK.

URBANA, ILLINOIS.



## PREFACE TO SECOND EDITION.

The kind reception accorded this volume by the instructors in physiological chemistry in the United States and Great Britain has made the preparation of a new edition imperative, notwithstanding the fact that less than two years have elapsed since the former edition appeared. The advance and development made in the field of physiological chemistry during this period have been both rapid and important; conditions which would of themselves have necessitated the revision of the volume at an early date.

The book has been thoroughly revised in all department and in part rewritten, the system of spelling officially adopted by the American Chemical Society having been followed throughout the volume. Besides introducing many new qualitative tests and quantitative methods, the author has added a chapter on "Enzymes and Their Action" and has rewritten the two chapters on Proteins. The term "protein" has been substituted for "proteid" and the classification of proteins as recently adopted by the American Physiological Society and the American Society of Biological Chemists has been introduced and is followed throughout the text; the classification adopted by the Chemical and Physiological Societies of England is also included.

The original plan of the book has been adhered to with the exception that the chapter on "Enzymes and Their Action" has been made Chapter I and the practical work upon the proteins is preceded by a chapter giving a brief discussion of protein substances from the standpoint of their decomposition and synthesis. We believe that the student will be able to pursue his practical work more intelligently and will derive greater benefit therefrom if the plan of instruction as suggested in Chapters IV and V be followed in the presentation of the subject of "Proteins."

The author wishes to express his thanks to all those who so kindly offered suggestions for the betterment of the book. He is particularly desirous of expressing his gratitude to Professor Lafayette B. Mendel and Dr. Thomas B. Osborne for the many helpful suggestions they have so kindly given him. His thanks are also due Professor C. A. Herter, Dr. H. D. Dakin, Dr. S. R. Benedict, and Mr. S. C. Clark for permission to insert unpublished material, to Mr. Paul E. Howe

for valuable assistance rendered in the reading of proof and in the verification of tests and methods, and to Dr. M. E. Rehfuss for assistance in proof reading.

The author takes this opportunity of making an acknowledgment which was inadvertently omitted from the first edition. He wishes to express his obligation to the laboratories of physiological chemistry at Yale University and at Columbia University (College of Physicians and Surgeons) in the latter of which he was Assistant to Professor W. J. Gies for two years. The courses given in these laboratories formed the basis of many of the experiments included in this volume, and it is with feelings of deepest gratitude that he records this acknowledgment of the assistance thus rendered by those in charge of these courses.

PHILIP B. HAWK.

URBANA, ILLINOIS.

## PREFACE TO FIRST EDITION.

The plan followed in the presentation of the subject of this volume is rather different, so far as the author is aware, from that set forth in any similar volume. This plan, however, he feels to be a logical one and has followed it with satisfactory results during a period of three years in his own classes at the University of Pennsylvania. The main point in which the plan of the author differs from those previously proposed is in the treatment of the food stuffs and their digestion.

In Chapter IV the "Decomposition Products of Proteids" has been treated although it is impracticable to include the study of this topic in the ordinary course in practical physiological chemistry. For the specimens of the decomposition products, the crystalline forms of which are reproduced by original drawings or by microphotographs, the author is indebted to Dr. Thomas B. Osborne of New Haven, Conn.

Because of the increasing importance attached to the examination of feces for the purposes of diagnosis, the author has devoted a chapter to this subject. He feels that a careful study of this topic deserves to be included in the courses in practical physiological chemistry, of medical schools in particular. The subject of *solid tissues* (Chapters XIII, XIV and XV) has also been somewhat more fully treated than has generally been customary in books of this character.

The author is deeply indebted to Professor Lafayette B. Mendel, of Yale University, for his careful criticism of the manuscript and to Professor John Marshall, of the University of Pennsylvania, for his painstaking revision of the proof. He also wishes to express his gratitude to Dr. David L. Edsall for his criticism of the clinical portion of the volume; to Dr. Otto Folin for suggestions regarding several of his quantitative methods, and to Mr. John T. Thomson for assistance in proof reading.

For the micro-photographs of oxyhæmoglobin and hæmin reproduced in Chapter XI the author is indebted to Professor E. T. Reichert, of the University of Pennsylvania, who, in collaboration with Professor A. P. Brown, of the University of Pennsylvania, is making a very extended investigation into the crystalline forms of biochemic substances. The micro-photograph of allantoin was kindly furnished by Professor Mendel.

The author is also indebted for suggestions and assistance received from the lectures and published writings of numerous authors and investigators.

The original drawings of the volume were made by Mr. Louis Schmidt whose eminently satisfactory efforts are highly appreciated by the author.

PHILIP B. HAWK.

PHILADELPHIA.

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# PHYSIOLOGICAL CHEMISTRY.

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## CHAPTER I.

### ENZYMES AND THEIR ACTION.

According to the old classification ferments were divided into two classes, the *organized ferments* and the *unorganized ferments*. As organized ferments or true ferments there were grouped such substances as *yeast* and certain *bacteria* which were supposed to act by virtue of vital processes, whereas the unorganized ferments included *salivary amylase* (ptyalin), *gastric protease* (pepsin), *pancreatic protease* (trypsin), etc., which were described as "non-living unorganized substances of a chemical nature." Kühne designated this latter class of substances as *enzymes* (*ἐν ζύμῃ*—in yeast). This division into organized ferments (true ferments) and unorganized ferments (enzymes) was generally accepted and was practically unquestioned until Buchner overthrew it in the year 1897 by his epoch-making investigations on zymase. Previous to this time many writers had expressed the opinion that the action of the ferment organisms was similar to that of the unorganized ferments or enzymes and that therefore the activity of the former was possibly due to the production of a substance in the cell, which was in nature similar to an enzyme. Investigation after investigation, however, failed to isolate any such principle from an active cell and the exponents of the "vital" theory became strengthened in their belief that certain fermentative processes brought about by living cells could not occur apart from the biological activity of such cells. However, as early as 1858, Traube had enunciated, in substance, the principles which were destined to be fundamental in our modern theory of fermentation. He expressed the belief that the yeast cell produced a product in its metabolic activities which had the property of reacting with sugar with the production of carbon dioxide and alcohol, and further that this reaction between the product of the metabolism of the yeast cell and the sugar occurred *without aid from the original cell*. It was not until 1897, however, that this theory was placed upon a firm experimental basis. This was brought about through the efforts of Buchner who succeeded in isolating from the living yeast cells a substance (zymase) which, when freed from the last trace of organized cellular material, was able to bring about the identical fermentative processes, which, up to this time, had been deemed possible only in the presence of the active, living yeast cell.

Buchner's manipulation of the yeast cells consisted in first grind-

ing them with sand and infusorial earth, after which the finely divided material was subjected to great pressure (300 atmospheres) and yielded a liquid which possessed the fermentative activity of the unchanged yeast cell.<sup>1</sup> This liquid contained *zymase*, the principal enzyme of the yeast cell. Later the lactic-acid- and acetic-acid-producing bacteria were subjected by Buchner to treatment similar to that accorded the yeast cells, and the active intracellular enzymes were obtained. Many other instances are on record in which a soluble, active agent has been isolated from ferment cells, with the result that it is pretty well established that all the so-called organized ferments elaborate substances of this character. Therefore, basing our definition on the work of Buchner and others we may define an enzyme as *an unorganized, soluble ferment, which is elaborated by an animal or vegetable cell and whose activity is entirely independent of any of the life processes of such a cell*. According to this definition the enzyme *zymase* elaborated by the yeast cell is entirely comparable to the enzyme *pepsin* elaborated by the cells of the stomach mucosa. One is derived from a vegetable cell, the other from an animal cell, yet the activity of neither is dependent upon the integrity of the cell.

Enzymes act by *catalysis* and hence may be termed catalyzers or catalysts. A simple rough definition of a catalyzer is "a substance which alters the velocity of a chemical reaction without undergoing any apparent physical or chemical change itself and without becoming a part of the product formed." It is a well-known fact that the velocity of the greater number of chemical reactions may be changed through the presence of some catalyzer. For example, take the case of hydrogen peroxide. It spontaneously decomposes slowly into the water and oxygen. In the presence of colloidal platinum,<sup>2</sup> however, the decomposition is much accelerated and ceases only when the destruction of the hydrogen peroxide is complete. Without multiplying instances, suffice it to say that there is an analogy between inorganic catalyzers and enzymes, the main point of difference between the enzymes and most of the inorganic catalyzers being that the enzymes are colloids.<sup>3</sup>

Inasmuch as each of the enzymes has an action which is more or less specific in character, and since it is a fairly simple matter, ordinarily, to determine the character of that action, the classification of the enzymes is not attended with very great difficulties. They are ordinarily classified according to the nature of the substrate<sup>4</sup> or according to the type

<sup>1</sup> In later investigations the process was improved by freezing the ground cells with liquid air and finely pulverizing them before applying the pressure.

<sup>2</sup> Produced by the passage of electric sparks between two platinum terminals immersed in distilled water, thus liberating ultra-microscopic particles.

<sup>3</sup> Bredig has been able to obtain certain inorganic catalyzers in *colloidal solution*. These he calls "*inorganic enzymes*."

<sup>4</sup> Substance acted upon. See Lippmann: *Ber. d. Deutsch. Chem. Ges.*, 36, 331, 1903.

of reaction they bring about. Thus we have various classes of enzymes, such as *amylolytic*,<sup>1</sup> *proteolytic*, *lipolytic*, *glycolytic*, *uricolytic*, *autolytic*, *oxidizing*, *reducing*, *inverting*, *protein-coagulating*, *deamidizing*, etc. In every instance the class name indicates the individual type of enzymatic activity which the enzymes included in that class are capable of accomplishing. For example, amylolytic enzymes facilitate the hydrolysis of starch (*amylum*) and related substances, lipolytic enzymes facilitate the hydrolysis of fats (*λίπος*), whereas through the agency of uricolytic enzymes uric acid is broken down. There is a tendency, at the present time, to harmonize the nomenclature of the enzymes by the use of the termination, *-ase*. According to this system of nomenclature, all starch-transforming enzymes, or so-called amylolytic enzymes, are called *amylases*, all fat-splitting enzymes are called *lipases*, etc. Thus *ptyalin* the amylolytic enzyme of the saliva, would be termed *salivary amylase* in order to distinguish it from *pancreatic amylase* (*amylopsin*) and *vegetable amylases* (*diastase*, etc.). According to the same system, the fat-splitting enzyme of the gastric juice would be termed *gastric lipase* to differentiate it from *pancreatic lipase* (*steapsin*), the fat-splitting enzyme of the pancreatic juice.

Euler<sup>2</sup> claims that enzymatic *cleavage* and *synthesis* are often brought about by two different components of an enzyme preparation. He would indicate this fact by giving the termination *-ese* to those enzymes exerting a synthetic function. For example, the enzyme which catalyzes the formation of nitriles Euler would call *nitrilase* in distinction from *nitrilase* which splits nitriles. He would further designate as *phosphatase* the enzyme which builds up phosphoric acid esters of carbohydrates in distinction from *phosphatase* which causes their cleavage. In the same way he would differentiate the lipolytic enzymes into *lipases* and *lipeses*.

Our knowledge regarding the distribution of enzymes has been wonderfully broadened in recent years. Up to within a few years, the real scientific information as to the enzymes of the animal organism, for example, was limited, in the main, to a rather crude understanding of the enzymes intimately connected with the main digestive functions of the organism. We now have occasion to believe that enzymes are doubtless present in every animal cell and are actively associated with all vital phenomena. As a preëminent example of such cellular activity may be cited the liver cell with its reputed complement of 15-20 or more enzymes.

<sup>1</sup> Armstrong suggests the use of the termination "clastic" instead of "lytic." He calls attention to the fact that *amylolytic*, in analogy with *electrolytic*, means "decomposition by means of starch" and is therefore a misnomer. He suggests the use of *amylolclastic*, *proteolclastic*, etc.

<sup>2</sup> Euler: *Zeitschrift für physiologische chemie*, 74, 13, 1911.

A list of the more important enzymes together with their *class, distribution, substrate* and *end-products* is given below.

## CLASSIFICATION OF ENZYMES.

Name.	Class.	Distribution.	Substrate.	End-products.
<i>Adenase</i> .....	Deamidizing.....	Animal tissues....	Adenine.....	Hypoxanthine.
<i>Amylases</i> .....	Amylolytic.....			
(a) <i>Pancreatic</i> ..... (amylopsin)		Pancreatic juice....	Starch, dextrine, etc.	Maltose.
(b) <i>Salivary</i> ..... (ptyalin)		Saliva.....	Starch, dextrine, etc.	Maltose.
(c) <i>Vegetable</i> ..... (diastase)		Plant tissues.....	Starch, dextrine, etc.	Maltose.
<i>Arginase</i> .....	Arginine-splitting.	Mucosa of intestine and in parenchyma of liver, kidney, spleen, etc.	Arginine.....	Ornithine and urea.
<i>Bromelin</i> .....	Proteolytic.....	Pineapple.....	Proteins.....	Proteoses, peptones, etc.
<i>Carboxylase</i> .....	Decarboxylizing.	Yeast.....	COOH group of aliphatic acids.	Carbon dioxide.
<i>Catalase</i> .....	Oxidizing.....	Tissues.....	Peroxides.....	Oxygen, water.
<i>Emulsin</i> (synaptase).	Glucoside-splitting.	Plants.....	Amygdalin, etc....	Glucose, etc.
<i>Enterokinase</i> .....	Activating.....	Intestinal epithelium.	Trypsinogen.....	Trypsin.
<i>Erepsin</i> (protease).	Proteolytic.....	Intestinal mucosa of man and dogs. Animal and vegetable tissues, and pancreatic juice.	Proteoses, peptones, peptides, and casein.	Simple cleavage products, such as amino acids.
<i>Glycogenase</i> .....	Glycogen-splitting.	Liver, intestinal mucosa (?), muscles (?).	Glycogen.....	Maltose and dextrin (dextrose?).
<i>Glycolytic enzymes</i>	Glycolytic.....	Blood and various organs.	Sugar.....	Lactic acid, alcohol, carbon dioxide and water.
<i>Guanase</i> .....	Deamidizing....	Animal tissues....	Guanine.....	Xanthine.
<i>Laccase</i> .....	Oxidizing.....	Sap of lac tree and other saps and juices; fungi; gum arabic, etc.	Polyhydric <i>p</i> -phenols such as hydroquinone and pyrogallol.	Oxidation products.
<i>Lactase</i> .....	Lactose-splitting.	Intestinal juice and mucosa.	Lactose.....	Dextrose and galactose.
<i>Lipases</i> .....	Lipolytic.....			
(a) <i>Pancreatic</i> ..... (steapsin)		Pancreatic juice....	Neutral fats.....	Fatty acid and alcohol.
(b) <i>Gastric</i> .....		Gastric juice.....	Neutral fats.....	Fatty acid and alcohol.
(c) <i>Vegetable</i> .....		Plant tissues.....	Neutral fats.....	Fatty acid and alcohol.
(d) <i>Animal</i> .....		Animal tissues....	Neutral fats.....	Fatty acid and alcohol.
<i>Maltase</i> .....	Maltose-splitting.	Blood serum, liver, saliva, pancreatic and intestinal juice and lymph.	Maltose.....	Dextrose.
<i>Nuclease</i> .....	Nucleoprotein hydrolyzing.	Tissues.....	Nucleoprotein.....	Purine bases.
<i>Oxidases</i> .....	Oxidizing.....	Plant and animal tissues.	Various tissue constituents.	Oxidation products.
<i>Pancreatic rennin</i> ..	Coagulating.....	Pancreatic juice....	Caseinogen.....	Casein.



CLASSIFICATION OF ENZYMES.—*Continued.*

Name.	Class.	Distribution.	Substrate.	End-products.
Papain (papayotin)	Proteolytic . . . . .	Pawpaw . . . . .	Proteins . . . . .	Proteoses, peptones, etc.
<i>Pepsin</i> (pepsase or acid-proteinase).	Proteolytic . . . . .	Gastric juice . . . . .	Proteins . . . . .	Proteoses, peptones and peptides.
Peroxidases . . . . .	Oxidizing . . . . .	Plant and animal tissues.	Peroxides, or hydroperoxides and carries oxygen to tissue constituents.	Oxidation products.
Phytase . . . . .	Phytin-splitting . . . . .	Rice bran . . . . .	Phytin . . . . .	Inosite and phosphoric acid.
Protease (erepsin) . . . . .	Proteolytic . . . . .	Kachree gourd . . . . .	Proteins . . . . .	Proteoses, peptones, peptides, etc.
<i>Rennin</i> (rennase or caseinase.)	Coagulating . . . . .	Gastric and pancreatic juices.	Caseinogen . . . . .	Casein.
<i>Sucrase</i> ( <i>invertase</i> or invertin).	Inverting . . . . .	Mucosa and juice of the intestine.	Sucrose . . . . .	Dextrose and Lævulose (invert-sugar).
<i>Thrombin</i> . . . . .	Coagulating . . . . .	Blood . . . . .	Fibrinogen . . . . .	Fibrin.
Trypsin (trypsaase or alkali-proteinase.)	Proteolytic . . . . .	Pancreatic juice . . . . .	Proteins . . . . .	Proteoses, peptones, peptides and amino acids.
Tyrosinase . . . . .	Oxidizing . . . . .	Plant and animal tissues.	Tyrosine . . . . .	Homogentisic acid, etc.
<i>Urease</i> . . . . .	Urea-splitting . . . . .	<i>Micrococcus ureæ</i> . . . . .	Urea . . . . .	Carbon dioxide and ammonia.
Uricase (uricolytic enzyme).	Uric acid-splitting . . . . .	Tissues . . . . .	Uric acid . . . . .	Allantoin, urea, glycooll and glyoxylic acid (?).
Xantho-oxidase . . . . .	Oxidizing . . . . .	Tissues . . . . .	Xanthine and hypoxanthine.	Uric acid.
<i>Zymase</i> . . . . .	Sugar-fermenting . . . . .	Yeast . . . . .	Sugar . . . . .	Alcohol and carbon dioxide.
<i>Inulase</i> . . . . .	Hydrolytic . . . . .	Plants and fungi . . . . .	Inulin . . . . .	Lævulose.
Rhamnase . . . . .	Hydrolytic . . . . .	Fungi . . . . .	Rhamnose . . . . .	
Trehalase . . . . .	Hydrolytic . . . . .	Fungi . . . . .	Trehalose . . . . .	

In text-book discussions of the enzymes it is customary to say that very little is known regarding the chemical characteristics of these substances since no member of the enzyme group has, up to the present time, been prepared in an *absolutely pure condition*. Apparently, however, from the nature of the facts in the case, it would be much more accurate to say that we absolutely *do not know* whether a specific enzyme *has, or has not*, been prepared in a pure state. (Some authors, like Arthus, have assumed that enzymes are not chemical individuals, but *properties conferred upon bodies*.) The enzymes are very difficult to prepare in anything like a condition approximating purity, since they are very prone to change their nature during the process by which the investigator is attempting to isolate them. For this reason we have absolutely no proof that the final product obtained is, or is not, in the

same state of purity it possessed in the original cell. Some of the enzymes are more or less closely associated with the proteins from the fact that they are both formed in every cell as the result of the cellular activity, both may be removed from solution by "salting-out," both are for the most part non-diffusible and are probably very similar as regards elementary composition. Hence in the preparation of some enzymes it is extremely difficult to make an absolute separation from the protein.<sup>1</sup> Under certain conditions enzymes are readily adsorbed by shredded protein material, such as fibrin, and may successfully resist the most prolonged attempts at washing them free. We may summarize some of the properties of the great body of enzymes as follows: Enzymes are soluble in *dilute* glycerol, sodium chloride solution, *dilute* alcohol and water, and precipitable by ammonium sulphate and *strong* alcohol. Their presence may be proven from the nature of the end-products of their action and not through the agency of any chemical test. They are *colloidal* and *non-diffusible*, and occur closely associated with protein material with which they possess many properties in common. Each enzyme shows the greatest activity at a certain temperature called the *optimum* temperature; there is also a *minimum* and a *maximum* temperature for each specific enzyme. Their action is inhibited by sufficiently lowering the temperature, and the enzyme, if in solution, is entirely destroyed by subjecting it to a temperature of 100° C. The best known enzymes, whether derived from warm-blooded or cold-blooded animals, are most active between 35°–45° C. The nature of the surrounding media alters the velocity of the enzymatic action, some enzymes being more active in acid solution whereas others require an alkaline fluid.

Many of the more important enzymes do not occur preformed within the cell, but are present in the form of a *zymogen* or mother-substance. In order to yield the active enzyme this zymogen must be transformed in a certain specific manner and by a certain specific substance. This transformation of the inactive zymogen into the active enzyme is termed *activation*. For instance, the zymogen of the enzyme pepsin of the gastric juice, termed *pepsinogen*, is activated by the *hydrochloric acid* secreted by the gastric cells (see p. 127), whereas the activation of the *trypsinogen* of the pancreatic juice is brought about by a substance termed *enterokinase*<sup>2</sup> (see p. 15). These are examples of many well-known activation processes going on continually within the animal organism. The agency which is instrumental in activating a zymogen is generally termed a *zymo-exciter* or a *kinase*. In the cases

<sup>1</sup> Others seem to be like the substrate on which they act, *e. g.*, carbohydrate.

<sup>2</sup> According to Delezenne, trypsinogen may be rapidly activated by soluble calcium salts

cited hydrochloric acid would be termed a zymo-exciter and entero-kinase would be termed a kinase.

After filtering yeast juice, prepared by the Buchner process (see p. 1), through a Martin gelatin filter, Harden and Young showed that the colloids left behind and the filtrate were both inactive fermentatively. Upon treating the colloid material (enzyme) with some of the filtrate, however, the mixture was shown to be able to bring about pronounced fermentation. It is believed that a *co-enzyme* present in the filtrate was the efficient agent in the transformation of the inactive enzyme. It is necessary to make frequent renewals of the co-enzyme in order to maintain continuous fermentation. It was further shown that this co-enzyme, in addition to being diffusible, was not destroyed by boiling and that it disappeared from yeast juice when this latter was fermented or allowed to undergo autolysis. The exact nature of this co-enzyme of zymase is unknown. The co-enzyme action, in this case, is probably dependent upon the presence of two individual agencies, one of which is *phosphates*.

It has been shown by Loevenhart that the property of acting as a pancreatic lipase co-enzyme is vested in *bile salts*, and Magnus has further shown that the synthetic salts are as efficient in this regard as the natural ones. A few other instances of co-enzyme demonstrations have been reported.

Electrolytes are very important factors in facilitating, or inhibiting enzyme action.<sup>1</sup> For example, magnesium hydroxide inhibits the action of salivary amylase<sup>2</sup> whereas the Cl ion facilitates the action of this and other amylases.<sup>3</sup> In fact Bierry<sup>4</sup> has very recently gone so far as to assert that the presence of the Cl or Br ion is "absolutely essential to the activity of pancreatic amylase."

The so-called "specificity" of enzyme action is an interesting and important fact. That enzymes are very specific as to the character of the *substrate*, or substance acted upon, is well known. Emil Fischer investigated this problem of specificity extensively in connection with the fermentation of sugars and reached the conclusion that enzymes, with the possible exception of certain oxidases, can act only upon such substances as have a specific stereo-isomeric relationship to themselves. He considers that the enzyme and its substrate must have an inter-relation, *such as the key has to the lock*, or the reaction does not occur. Fischer was able to predict, in certain definite cases, from a knowledge of the constitution and stereo-chemical relationships of a substance,

<sup>1</sup> For literature, see Kendall and Sherman: *Jour. Am. Chem. Soc.*, 32, 1087, 1910.

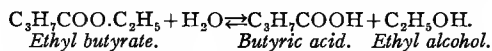
<sup>2</sup> Bergeim and Hawk: Unpublished data.

<sup>3</sup> Wohlgenuth: *Biochemische Zeitschrift*, 9, 10, 1908.

<sup>4</sup> Bierry: *Ibid.*, 40, 357, 1912.

whether or not it would be acted upon by a certain enzyme. An application of this specificity of enzyme action may be seen in the well-known facts that certain enzymes act on carbohydrates, others on fats, and others on protein; and, moreover, that the group of those which transform carbohydrates, for example, is further subdivided into specific enzymes each of which has the power of acting alone upon some one sugar.

It has been conclusively shown, in the case of certain enzymes,<sup>1</sup> at least, that their action is a *reversible* one and is, in all its main features, directly analogous to the reversible reactions produced by chemical means. For instance, in the saponification of ethyl-butyrate by means of pancreatic lipase, it has been shown that upon the formation of the end-products of the reaction, *i. e.*, butyric acid and ethyl alcohol, there is reversion<sup>2</sup> and the reaction is stationary. This does not mean



there are no chemical changes going on, but simply indicates that chemical *equilibrium* has been established, and that the change in one direction is counterbalanced by the change in the opposite direction. Pancreatic lipase was one of the first enzymes to have the reversibility of its reaction clearly demonstrated.<sup>3</sup> A knowledge of the fact that lipase possesses this reversibility of action is of extreme physiological importance and aids us materially in the explanation of the processes involved in the digestion, absorption, and deposition of fats in the animal organism (see p. 141).

In respect to many enzymes it has been found that the law governing the action of inorganic catalyzers is directly applicable, *i. e.*, that the *intensity is almost directly proportional to the concentration of the enzyme*. In the case of enzymes, however, there is a difference in that a maximum intensity is soon reached and that subsequent concentration of the enzyme is productive of no further increase in intensity. The enzymes which have been shown to obey this *linear law* are lipase, invertase, rennin, and trypsin. In *certain instances*, where this law of direct proportionality between the intensity of action and the concentration of enzyme does not hold, it has been found that the *Schütz-Borissow law*; first experimentally demonstrated by E. Schütz, was applicable. This is to the effect that the intensity is directly proportional to the square root of the concentration, or conversely, that *the relative concentrations of enzymes are directly proportional to the squares of the intensities*.<sup>4</sup>

<sup>1</sup> This is probably a general condition.

<sup>2</sup> The re-synthesis of ethyl-butyrate from its hydrolysis products. This may be indicated thus:

<sup>3</sup> The principle was *first* demonstrated in connection with the enzyme maltase (see p. 62).

<sup>4</sup> This Schütz-Borissow law is not generally applicable.

It has been shown that there are certain substances which possess the property of directly inhibiting or preventing the action of a catalyzer. These are called *anti-catalyzers* or paralyzers and have been compared to the *anti-toxins*. Related to this class of anti-catalytic agents stand the *anti-enzymes*. The first anti-enzyme to be reported was the *anti-rennin* of Morgenroth. This was produced by injecting into an animal increasing doses of rennet solution, whereupon an "anti" substance was subsequently found both in the serum and in the milk, which prevented the enzyme rennin from exerting its normal activity in the presence of caseinogen. In other words, *anti-rennin* had been formed in the serum of the animal,<sup>1</sup> through the repeated injections of rennet solution. Since the discovery of this anti-enzyme, anti-bodies have been demonstrated for pepsin, trypsin, lipase, urease, amylase, laccase, tyrosinase, emulsin, papain, and thrombin. According to Weinland, the reason why the stomach does not digest itself is, that during life there is present in the mucous membrane of the stomach an *anti-enzyme* (*anti-pepsin*) which has the property of inhibiting the action of pepsin. A similar substance (anti-trypsin) is present in the intestinal mucosa as well as in the tissues of various intestinal worms. Some investigators are not inclined to accept the enzyme nature of these inhibitory agents as proven.

The very recent investigations of Ehrlich<sup>2</sup> and of Neuberg<sup>3</sup> have served to cause a complete revision of our ideas regarding yeast fermentation. Ehrlich, for example, has shown that *yeast will liberate ammonia from amino acids* and leave behind a non-nitrogenous complex. Among these complexes amyl alcohol, succinic acid and others may be mentioned. Thus, *amyl alcohol* results from the fermentation of leucine, whereas ethyl alcohol results from the fermentation of sugar. Neuberg has demonstrated the presence in the yeast of an enzyme termed *carboxylase* which has the property of *splitting off carbon dioxide from the carboxyl group of amino and other aliphatic acids*. The findings mentioned above constitute the basis for much important work on so-called "sugar-free fermentation."

For a more extended consideration of enzymes the student is referred to the following sources:—

BAYLISS.—The Nature of Enzyme Action, Second Edition, Longmans, Green and Co., New York and London.

DUCLAUX.—Traité de Microbiologie, Masson & Co., Paris.

EFFRONT.—Enzymes and their Applications, Translated by Prescott, Wiley and Sons, New York.

<sup>1</sup> Serum is normally *anti-tryptic*.

<sup>2</sup> Ehrlich: *Biochemische Zeitschrift*, 36, 477, 1911.

<sup>3</sup> Neuberg and Collaborators: *Biochemische Zeitschrift*, 31, 170; 32, 323; 36 (60, 68, and 76), 1911.

EULER.—(a) Allgemeine Chemie der Enzyme, Bergmann, Wiesbaden, 1910. (b) Ergebnisse der Physiologie, 1909–10.

OPPENHEIMER.—Die Fermente und Ihre Wirkungen, Dritte Auflage, Vogel, Leipzig.

SAMUELY.—Handbuch der Biochemie des Menschen und der Thiere (Oppenheimer), Gustav Fischer, Jena.

VERNON.—Intracellular Enzymes, Murray, London.

## EXPERIMENTS ON ENZYMES AND ANTI-ENZYMES.

### A. Experiments on Enzymes.<sup>1</sup>

#### I. AMYLASES.

1. **Demonstration of Salivary Amylase.**<sup>2</sup>—To 25 c.c. of a one per cent starch paste in a small beaker, add 5 drops of saliva and stir thoroughly. At intervals of a minute remove a drop of the solution to one of the depressions of a test-tablet and test by the iodine test.<sup>3</sup> If the blue color with iodine still forms after five minutes, add another five drops of saliva. The opalescence of the starch solution should soon disappear, indicating the formation of *soluble starch (amidulin)* which gives a blue color with iodine. This body should soon be transformed into *erythrodextrin* which gives a red color with iodine, and this, in turn, should pass into *achroodextrin* which gives *no color* with iodine. This point is called the *achromic point*. When this point is reached test by Fehling's test<sup>4</sup> to show the production of a reducing substance (maltose). A positive Fehling's test may be obtained while the solution still reacts red with iodine inasmuch as some sugar is formed from the soluble starch coincidentally with the formation of the erythrodextrin. For further discussion of the transformation of starch see p. 61.

2. **Demonstration of Pancreatic Amylase.**<sup>5</sup>—Proceed exactly as indicated above in the Demonstration of Salivary Amylase except that the saliva is replaced by 5 c.c. of pancreatic extract prepared as described on p. 153. Pancreatic amylase transforms the starch in a manner entirely analogous to the transformation resulting from the action of salivary amylase.

3. **Preparation of Vegetable Amylase.**—Extract finely ground malt with water, filter and subject the filtrate to alcoholic fermentation by means of yeast. When fermentation is complete filter off the yeast

<sup>1</sup> If it is deemed advisable by the instructor to give all the practical work upon enzymes at this point in the course, additional experiments will be found in Chapters III, VI and VIII.

<sup>2</sup> For a discussion of this enzyme see p. 60.

<sup>3</sup> See p. 50.

<sup>4</sup> See p. 32.

<sup>5</sup> For a discussion of this enzyme see p. 150.

and precipitate the amylase from the filtrate by the addition of alcohol. The precipitate may be filtered off and obtained in the form of a fine white powder.

4. **Demonstration of Vegetable Amylase.**—This enzyme may be demonstrated according to the directions given under Demonstration of Salivary Amylase, p. 10, with the exception that the saliva used in that experiment is replaced by an aqueous solution of the vegetable amylase powder prepared as described above.<sup>1</sup>

## II. PROTEASES.

1. **Preparation of Gastric Protease.**<sup>2</sup>—Treat the finely comminuted mucosa of a pig's stomach with 0.4 per cent hydrochloric acid and extract at 38° C. for 24–48 hours. The filtrate from this mixture constitutes a very satisfactory acid extract of gastric protease (see p. 130).

2. **Demonstration of Gastric Protease.**—Introduce some protein material (fibrin, coagulated egg-white, or washed lean beef) into the acid extract of gastric protease prepared as above described,<sup>3</sup> add an equal volume of 0.4 per cent hydrochloric acid and place the mixture at 38° C. for 2–3 days. Identify the products of digestion according to directions given on p. 130.

3. **Preparation of Pancreatic Protease.**<sup>4</sup>—A satisfactory extract of this enzyme may be made from the pancreas of a pig or sheep according to the directions given on p. 153.

4. **Demonstration of Pancreatic Protease.**—Into an alkaline extract of pancreatic protease,<sup>5</sup> prepared as directed on p. 153, introduce some fibrin, coagulated egg-white or lean beef and place the mixture at 38° C. for 2–5 days.<sup>6</sup> At the end of that period separate and identify the end-products of the action of pancreatic protease according to the directions given on p. 153.

5. **Demonstration of a Vegetable Protease.**—A commercial preparation of *papain* (*papayotin*, *carase* or *papase*), the protease of the fruit of the pawpaw (*carica papaya*), may be used in this connection. Follow the same procedure as that described under Gastric Protease (see above).

<sup>1</sup> If desired the *first* aqueous extract of the original malt may be used in this demonstration. Commercial *taka-diastase* may also be employed.

<sup>2</sup> Also called *pepsin*, *pepsase*, *gastric proteinase*, and *acid protease*. For a discussion of this enzyme see p. 127.

<sup>3</sup> If so desired, a solution of commercial pepsin powder in 0.2 per cent. hydrochloric acid may be substituted.

<sup>4</sup> Also called *trypsin*, *trypcase*, *pancreatic proteinase* and *alkali proteinase*. For a discussion of this enzyme see p. 149.

<sup>5</sup> A 0.25 per cent sodium carbonate solution of commercial *trypsin* may be substituted.

<sup>6</sup> A few c.c. of toluol or an alcoholic solution of thymol should be added to prevent putrefaction.

It has been demonstrated by Mendel and Blood<sup>1</sup> that the presence of HCN will accelerate the proteolytic activity of papain. It is suggested that the HCN acts as a so-called *co-enzyme* (see p. 7).

Vines<sup>2</sup> believes that "papain" consists of a mixture of two enzymes, a pepsin and an erepsin. Mendel and Blood do not consider the evidence on this point as conclusive.

### III. LIPASES.

1. **Preparation of Pancreatic Lipase.**<sup>3</sup>—An extract of this enzyme may be prepared from the pancreas of the pig or sheep according to the directions given on p. 153.<sup>4</sup>

2. **Demonstration of Pancreatic Lipase.**—Into each of two test-tubes introduce 10 c.c. of milk and a small amount of litmus powder. To the contents of one tube add 3 c.c. of a *neutral* extract of *pancreatic lipase* and to the contents of the other tube add 3 c.c. of a *boiled* neutral extract of *pancreatic lipase*. Keep the tubes at 38° C. and watch for color changes. The blue color of the litmus powder will gradually give place to a red. This change in color of the litmus from blue to red has been brought about by the fatty acid which has been produced through the lipolytic action exercised by the lipase upon the milk fats.

3. **Preparation of Vegetable Lipase.**—This enzyme may be readily prepared from castor beans, several months old, by the following procedure:<sup>5</sup> Grind the shelled beans very fine<sup>6</sup> and extract for twenty-four-hour periods with alcohol-ether and ether, in turn. Reduce the semi-fat-free material to the finest possible consistency by means of mortar and pestle and pass this material through a sieve of very fine mesh. Place this material in a Soxhlet extractor and extract for one week. This fat-free powder may then be used to demonstrate the action of vegetable lipase. Powder prepared as described may be used in quantitative tests. For ordinary qualitative tests it is not necessary to remove the last traces of fat and therefore the extraction period in the Soxhlet apparatus may be much shortened.

4. **Demonstration of Vegetable Lipase.**—The lipolytic action of the lipase prepared from the castor bean, as just described, may be demonstrated in a manner entirely analogous to that used in the Demonstration of Pancreatic Lipase, see above. Proceed as indicated

<sup>1</sup> Mendel and Blood: *Journal of Biological Chemistry*, 8, 177, 1910.

<sup>2</sup> Vines: *Annals of Botany*, 19, 174, 1905.

<sup>3</sup> Also called steapsin. For a discussion of this enzyme see p. 151. A very active lipolytic extract may also be prepared from the liver.

<sup>4</sup> If preferred, a *glycerol extract* may be prepared according to the directions given by Kanitz; *Zeitschrift für physiologische Chemie*, 1906, 46, p. 482.

<sup>5</sup> A. E. Taylor: *On Fermentation*; *University of California Publications*, 1907.

<sup>6</sup> The shells should be removed without the use of water. These beans are *poisonous*, due to their content of *ricin*.



in that experiment and substitute the vegetable lipase powder for the neutral extract of pancreatic lipase. The type of action is entirely analogous in the two instances.

An experiment similar to Experiment 2, p. 157, may also be tried if desired. In this experiment 0.2 c.c. of either *ethyl butyrate* or *amyl acetate* may be employed.

#### IV. INVERTASES.<sup>1</sup>

1. **Preparation of an Extract of Sucrase.**<sup>2</sup>—Treat the finely divided epithelium of the small intestine of a *dog*, *pig*, *rat*, *rabbit*, or *hen* with about three volumes of a two per cent solution of sodium fluoride and permit the mixture to stand at room temperature for twenty-four hours. Strain the extract through cloth or absorbent cotton and use the strained material in the following demonstration.

2. **Demonstration of Sucrase.**—To about 5 c.c. of a one per cent solution of sucrose, in a test-tube, add about one cubic centimeter of a two per cent sodium fluoride intestinal extract, prepared as described above. Prepare a control tube in which the intestinal extract is *boiled* before being added to the sugar solution. Place the two tubes at 38° C. for two hours.<sup>3</sup> Heat the mixture to boiling to coagulate the protein material, filter, and test the filtrate by Fehling's test (see p. 32). The tube containing the *boiled* extract should give no response to Fehling's test, whereas the tube containing the *unboiled* extract should reduce the Fehling's solution. This reduction is due to the formation of *invert sugar* (see p. 46) from the sucrose through the action of the enzyme *sucrase* which is present in the intestinal epithelium.

3. **Preparation of Vegetable Sucrase.**—Thoroughly grind about 100 grams of brewer's yeast in a mortar with sand. Spread the ground yeast in thin layers on glass or porous plates and dry it rapidly in a current of dry, warm air. Powder this dry yeast, extract it with distilled water and filter. Pour the filtrate into acetone, stir and after permitting the acetone mixture to stand for a few minutes filter on a Buchner funnel. The resulting precipitate, after drying and pulverizing, may be used to demonstrate vegetable sucrase.

4. **Demonstration of Vegetable Sucrase.**—To about 5 c.c. of a one per cent solution of sucrose in a test-tube add a small amount of the sucrase powder prepared as directed above. Place the tube at 38° C. for 24–72 hours and at the end of that period test the solution by Fehling's test. Reduction indicates that the active sucrase powder has transformed

<sup>1</sup> *The inverting enzymes of the alimentary tract*; Mendel and Mitchell: *American Journal of Physiology*, 20, 81, 1907–08.

<sup>2</sup> For a discussion of this enzyme see p. 152.

<sup>3</sup> If a positive result is not obtained in this time permit the digestion to proceed for a longer period.

the non-reducing sucrose into dextrose and lævulose, and these sugars, in turn, have reduced the Fehling solution.

5. **Preparation of an Extract of Lactase.**<sup>1</sup>—Treat the finely divided epithelium of the small intestine of a *kitten*, *puppy*, or *pig embryo* with about three volumes of a two per cent solution of sodium fluoride and permit the mixture to stand at room temperature for twenty-four hours. Strain the extract through cloth or absorbent cotton and use the strained material in the following demonstration.

6. **Demonstration of Lactase.**<sup>2</sup>—To about 5 c.c. of a one per cent solution of lactose in a test-tube add about one cubic centimeter of a toluol-water or a two per cent sodium fluoride extract of the first part of the small intestine<sup>3</sup> of a *kitten*, *puppy*, or *pig embryo* prepared as described above. Prepare a control tube in which the intestinal extract is *boiled* before being added to the sugar solution. Place the two tubes at 38° C. for 24 hours. At the end of this period add one cubic centimeter of the digestion mixture to 5 c.c. of Barfoed's<sup>4</sup> reagent and place the tubes in a boiling water-bath.<sup>5</sup> Examine the tubes at the end of three minutes against a black background in a good light. If no cuprous oxide is visible replace the tubes and repeat the examination at the end of the *fourth* and *fifth* minutes. If no reduction is then observed permit the tubes to stand at room temperature for 5–10 minutes and examine again.<sup>6</sup>

It has been determined that disaccharide solutions will not reduce Barfoed's reagent until after they have been heated for 9–10 minutes on a boiling water-bath in contact with the reagent.<sup>7</sup> Therefore in the above test, if the tube containing the *unboiled* extract exhibits any reduction after being heated as indicated, for a period of five minutes or less, and the control tube containing *boiled* extract shows no reduction, it may be concluded that *lactase* was present in the intestinal extract.<sup>8</sup>

7. **Preparation of an Extract of Maltase.**<sup>9</sup>—Treat the finely divided epithelium of the small intestine of a *cat*, *kitten*, or *pig (embryo or adult)* with about three volumes of a two per cent solution of sodium fluoride and permit the mixture to stand at room temperature for twenty-

<sup>1</sup> For a discussion of this enzyme see p. 152.

<sup>2</sup> Roaf; *Bio-Chemical Journal*, 3, 182, 1908.

<sup>3</sup> Duodenum and first part of jejunum.

<sup>4</sup> To 4.5 grams of neutral crystallized copper acetate in 900 c.c. of water, add 0.6 c.c. of glacial acetic acid and make the total volume of the solution one liter.

<sup>5</sup> Care should be taken to see that the water in the bath reaches at least to the upper level of the contents of the tubes.

<sup>6</sup> Sometimes the drawing of conclusions is facilitated by pouring the mixture from the tube and examining the bottom of the tube for adherent cuprous oxide.

<sup>7</sup> The heating for 9–10 minutes is sufficient to transform the disaccharide into monosaccharide.

<sup>8</sup> The reduction would, of course, be due to the action of the *dextrose* and *galactose* which had been formed from the lactose through the action of the enzyme *lactase*.

<sup>9</sup> For a discussion of this enzyme see p. 62.

four hours. Strain the extract through cloth and use the strained material in the following demonstration.

8. **Demonstration of Maltase.**—Proceed exactly as indicated in the demonstration of lactase, above, except that a one per cent solution of maltose is substituted for the lactose solution. The extract used may be prepared from the upper part of the intestine of a *cat*, *kitten*, or *pig* (*embryo* or *adult*). In the case of lactase, as indicated, the intestine used should be that of a *kitten*, *puppy*, or *pig* (*embryo*).

## V. EREPSIN.<sup>1</sup>

1. **Preparation of Erepsin.**—Grind the mucous membrane of the small intestine of a cat, dog, or pig with sand in a mortar. Treat the mortared membrane with toluol- or chloroform-water and permit the mixture to stand, with occasional shaking, for 24–72 hours.<sup>2</sup> Filter the extract thus prepared through cotton and use the filtrate in the following experiment.

2. **Demonstration of Erepsin.**—To about 5 c.c. of a one per cent solution of Witte's peptone in a test-tube add about 1 c.c. of the erepsin extract prepared as described above and make the mixture slightly alkaline (0.1 per cent) with sodium carbonate. Prepare a second tube containing a like amount of peptone solution but *boil* the erepsin extract before introducing it. Place the two tubes at 38° C. for 2–3 days. At the end of that period heat the contents of each tube to boiling, filter and try the biuret test on each filtrate. In making these tests care should be taken to use like amounts of filtrate, potassium hydroxide and copper sulphate in each test in order that the drawing of correct conclusions may be facilitated. The contents of the tube which contained the *boiled* extract should show a deep pink color with the biuret test, due to the peptone still present. On the other hand, the biuret test upon the contents of the tube containing the *unboiled* extract should be negative or exhibit, at the most, a *faint pink* or *blue color*, signifying that the peptone, through the influence of the erepsin, has been transformed, in great part at least, into *amino acids* which do not respond to the biuret test.<sup>3</sup>

3. **The Glycyl-Tryptophane Reaction.**—The dipeptide glycyl-tryptophane<sup>4</sup> may be used in place of the peptone solution for the demonstration of erepsin. It is used widely in the diagnosis of gastric cancer. It has been found that a peptide-splitting enzyme (erepsin) is

<sup>1</sup> For a discussion of this enzyme see p 152.

<sup>2</sup> The enzyme may also be extracted by means of *glycerol* or *alkaline* "physiological" salt solution if desired.

<sup>3</sup> *Strictly speaking*, this erepsin demonstration is not adequate unless a control test is made with *native protein* (except caseinogen, histones and protamines) to show that the extract is *trypsin-free* and digests peptone but not native protein.

<sup>4</sup> This dipeptide is sold commercially under the name "Ferment Diagnosticon."

present in the stomach contents of individuals suffering from cancer of the stomach, whereas the stomach contents of normal individuals contains no such enzyme. The glycyL-tryptophane test, therefore, furnishes a means of aiding in the diagnosis of this disorder. As applied to stomach contents, the test is as follows:<sup>1</sup> Introduce about 10 c.c. of the filtrate from the stomach contents into a test-tube, add a little glycyL-tryptophane, and a layer of toluol and place the tube in an incubator at 38° C. for 24 hours. At the end of this time by means of a pipette transfer 2-3 c.c. of the fluid from beneath the toluol to a test-tube, add a few drops of 3 per cent acetic acid and *carefully* introduce bromine vapors. Shake the tube and note the production of a red color if tryptophane is present. The tryptophane has, of course, been liberated from the peptide through the action of the peptide-splitting enzyme (erepsin) elaborated by the cancer tissue.

If an excess of bromine is added the color will vanish. If no rose color is noted, add more bromine vapors carefully with shaking until further addition of the vapors causes the production of a *yellowish* color. This indicates an excess of bromine and constitutes a negative test. Occasionally the rose color indicating a positive test is so transitory as to escape detection unless the test be *very* carefully performed.

## VI. URICOLYTIC ENZYME.<sup>2</sup>

1. **Preparation of Uricase (Uricolytic Enzyme).**—Extract pulped liver tissue with toluol- or chloroform-water at 38° C. for 24 hours, with occasional shaking. Filter the extract and use the filtrate in the following experiment.

2. **Demonstration of Uricase (Uricolytic Enzyme).**—Add about 0.1 gram of uric acid to 10 c.c. of water and bring the uric acid into solution by the addition of the minimal quantity of potassium hydroxide. To 5 c.c. of this uric acid solution, in a test-tube, add 5 c.c. of the uricolytic enzyme extract prepared as described above. Prepare a second tube containing a like amount of uric acid solution, but *boil* the extract before it is introduced. Place the two tubes at 38° C. for 3-4 days and titrate the two digestive mixtures with a solution of potassium permanganate according to directions given under Folin-Schaffer Method, Chapter XXII. It will be found that the mixture containing the *boiled* extract requires a much larger volume of the permanganate to complete the titration than the other tube. This indicates that a uricolytic enzyme has destroyed at least a portion of the uric acid which was originally present in the tube containing the *unboiled* extract.

<sup>1</sup> Neubauer and Fischer; *Deutsches Archiv f. klinische Medizin*, 97, 499, 1909.

<sup>2</sup> Mendel and Mitchell; *American Journal of Physiology*, 20, 97, 1908.

## VII. CATALASE.

**Demonstration of Catalase.**—The various animal tissues, such as *liver, kidney, blood, lung, muscle* and *brain*, contain an enzyme called *catalase* which possesses the property of decomposing hydrogen peroxide. The presence of this enzyme may be demonstrated as follows: Introduce into a low, broad, wide-mouthed bottle some pulped liver tissue and a porcelain crucible containing *neutral* hydrogen peroxide.<sup>1</sup> Connect the bottle with a eudiometer filled with water, upset the crucible of hydrogen peroxide upon the liver pulp and note the collection of gas in the eudiometer. This gas is oxygen which has been liberated from the hydrogen peroxide through the action of the catalase of the liver tissue.

See p. 23 for a method for the quantitative determination of catalase based on the above principle.

## B. Experiments on Anti-Enzymes.

1. **Preparation of an Extract of Anti-Pepsin.**<sup>2</sup>—Grind up a number of intestinal worms (*ascaris*)<sup>3</sup> with quartz sand in a mortar. Subject this mass to high pressure, filter the resultant juice and treat it with alcohol until a concentration of sixty per cent is reached. If any precipitate forms it should be filtered off<sup>4</sup> and alcohol added to the filtrate until the concentration of alcohol is 85 per cent, or over. The anti-enzyme is precipitated by this concentration. Permit this precipitate to stand for twenty-four hours, then filter it off, wash it with 95 per cent alcohol, absolute alcohol, and ether, in turn, and finally dry the substance over sulphuric acid. The sticky powder which results may be used in this form or may be dissolved in water as desired and the aqueous solution used.<sup>5</sup>

2. **Demonstration of Anti-Pepsin.**<sup>6</sup>—Introduce into a test-tube a few fibrin shreds and equal volumes of pepsin-hydrochloric acid<sup>7</sup> and *ascaris* extract made as indicated above. Prepare a control tube in which the *ascaris* extract is replaced by water. Place the tubes at 38° C. Ordinarily in one hour the fibrin in the control tube will be completely digested. The fibrin in the tube containing the *ascaris* extract

<sup>1</sup> Mendel and Leavenworth; *American Journal of Physiology*, 21, 85, 1908.

<sup>2</sup> Anti-gastric-protease or anti-acid-protease.

<sup>3</sup> These may be readily obtained from pigs at a slaughter house.

<sup>4</sup> This precipitate consists of impurities, the anti-enzyme not being precipitated until a higher concentration of alcohol is reached.

<sup>5</sup> The original *ascaris* extract possesses much greater activity than either the powder or the aqueous solution.

<sup>6</sup> Martin H. Fischer; *Physiology of Alimentation*, 1907, p. 134.

<sup>7</sup> Made by bringing 0.015 gram of pepsin into solution in 7 c. c. of water and 0.23 gram of concentrated hydrochloric acid.

may, however, remain unchanged for days, thus indicating the inhibitory influence exerted by the anti-enzyme present in this extract.

3. **Preparation of an Extract of Anti-Trypsin.**<sup>1</sup>—The extract may be prepared from the intestinal worm, ascaris, according to the directions given on page 17.

4. **Demonstration of Anti-Trypsin.**—Introduce into a test-tube a few shreds of fibrin and equal volumes of an artificial tryptic solution<sup>2</sup> and the ascaris extract made as described on page 17. Prepare a control tube in which the ascaris extract is replaced by water. Place the two tubes at 38° C. Ordinarily the fibrin in the control tube will be completely digested in two hours. The fibrin in the tube containing the ascaris extract may, however, remain unchanged for days, thus indicating the inhibitory influence of the anti-enzyme.

Blood serum also contains *anti-trypsin*. This may be demonstrated as follows: Introduce equal volumes of serum and artificial tryptic solution (prepared as described above) into a test-tube and add a few shreds of fibrin. Prepare a *control* tube containing *boiled* serum. Place the two tubes at 38° C. It will be observed that the fibrin in the tube containing the *boiled* serum digests, whereas that in the other tube does not digest. The anti-trypsin present in the unboiled serum has exerted an inhibitory influence upon the action of the trypsin.

### C. Quantitative Applications.

1. **Quantitative Determination of Amylolytic Activity.**—*Wohl-gemuth's Method.*<sup>3</sup> Arrange a series of test-tubes with diminishing quantities of the enzyme solution under examination, introduce into each tube 5 c.c. of 1 per cent solution of soluble starch<sup>4</sup> and place each tube at once in a bath of ice-water.<sup>5</sup> When all the tubes have been prepared in this way and placed in the ice-water bath they are transferred to a water-bath or incubator and kept at 38° C. for from thirty minutes to an hour.<sup>6</sup>

<sup>1</sup> Anti-pancreatic-protease or anti-alkali-protease.

<sup>2</sup> Made by dissolving 0.04 gram of sodium carbonate and 0.015 gram of trypsin in 8 c.c. of water.

<sup>3</sup> Wohl-gemuth; *Biochemische Zeitschrift*, 9, 1, 1908.

<sup>4</sup> Kahlbaum's soluble starch is satisfactory. In preparing the 1 per cent. solution, the weighed starch powder should be dissolved in *cold* distilled water in a casserole and stirred until a homogeneous suspension is obtained. The mixture should then be heated, with constant stirring, until it is clear. This ordinarily takes about 8–10 minutes. A slightly opaque solution is thus obtained which should be cooled and made up to the proper volume before using.

<sup>5</sup> Ordinarily a series of six tubes is satisfactory, the volumes of the enzyme solution used ranging from 1 c.c. to 0.1 c.c. and the measurements being made by means of a 1 c.c. graduated pipette. Each tube should be placed in the ice-water bath as soon as the starch solution is introduced. It will be found convenient to use a small wire basket to hold the tubes.

<sup>6</sup> Longer digestion periods may be used where it is deemed advisable. If exceedingly weak solutions are being investigated, it may be most satisfactory to permit the digestion to extend over a period of 24 hours.

At the end of this digestion period the tubes are again removed to the bath of ice-water in order that the action of the enzyme may be stopped.

Dilute the contents of each tube, to within about one-half inch of the top, with water, add one drop of a N/10 solution of iodine and shake the tube and contents thoroughly. A series of colors ranging from *dark blue* through *bluish-violet* and *reddish-yellow* to *yellow*, will be formed.<sup>1</sup> The dark blue color shows the presence of unchanged starch, the bluish-violet indicates a mixture of starch and erythrodextrin, whereas the reddish-yellow signifies that erythrodextrin and maltose are present and the yellow solution denotes the complete transformation of starch into maltose. Examine the tubes carefully before a white background and select the last tube in the series which shows the entire absence of all blue color, thus indicating that the starch has been completely transformed into dextrins and sugar. In case of indecision between two tubes, add an extra drop of the iodine solution, and observe them again, after shaking.

*Calculation.*—The amylolytic activity<sup>2</sup> of a given solution is expressed in terms of the activity of 1 c.c. of such a solution. For example, if it is found that 0.02 c.c. of an amylolytic solution, acting at 38° C., completely transformed the starch in 5 c.c. of a 1 per cent starch solution in 30 minutes, the amylolytic activity of such a solution would be expressed as follows:

$$D_{30}^{38} = 250.$$

This indicates that 1 c.c. of the solution under examination possesses the power of completely digesting 250 c.c. of 1 per cent starch solution in 30 minutes at 38° C.

Wohlgemuth has suggested a slight alteration in the above procedure for use in the determination of the amylase content of the feces.<sup>3</sup> A modification of the Wohlgemuth procedure<sup>4</sup> for this purpose is given in the latter part of the chapter on Feces.

2. **Quantitative Determination of Peptic Activity.**—(a) *Mett's Method.*—The determination of the actual rate of peptic activity is a most important procedure under certain conditions. Several methods of making this determination are in use. The method of Sprigg<sup>5</sup> is probably the most accurate method yet devised for this purpose. It is, however, too complicated and time-consuming for clinical purposes. The method of Mett, given below, is very simple although not strictly accurate. The procedure is as follows: To about 5 c.c. of the gastric juice under exami-

<sup>1</sup> See p. 61.

<sup>2</sup> Designated by "D" the first letter of "diastatic."

<sup>3</sup> Wohlgemuth; *Berliner klinische Wochenschrift*, 47, 92, 1910.

<sup>4</sup> Hawk; *Archives of Internal Medicine*, 8, 552, 1911.

<sup>5</sup> Sprigg; *Zeitschrift für physiologische Chemie*, 35, 465, 1902.

nation in a test-tube add 1-3 sections of a Mett tube<sup>1</sup> and place the mixture at 38° C. for *ten hours*. At the end of this period, the tube should be removed from the gastric juice and the length of the column of coagulated albumin which has been digested carefully determined by means of a low-power microscope and a millimeter scale. In normal human gastric juice the upper limit is 4 mm. However, control tests should always be made to determine the digestibility of the coagulated albumin in artificial gastric juice, inasmuch as this factor will vary with different albumin preparations. This fact of the variation in the digestibility has been emphasized by the recent work of Frank.<sup>2</sup> This investigator demonstrated that the digestibility of the egg albumin in the Mett tube would vary according to the temperature at which the albumin was coagulated. Therefore in making a series of comparative tests the albumin in the Mett tubes should be coagulated under uniform conditions in order to insure accuracy.

In connection with this test the Schütz-Borissow law should be borne in mind. This principle is to the effect that *the amount of proteolytic enzyme present in a digestion mixture is proportional to the square of the number of millimeters of albumin digested*. Therefore a gastric juice which digests 2 mm. of albumin contains *four times* as much pepsin as a gastric juice which digests only 1 mm. of albumin. And further, if the quantities of albumin digested are 2 mm. and 3 mm., respectively, the ratio between the pepsin values will be as 4 : 9.

It is claimed by Nirenstein and Schiff<sup>3</sup> that the principle of Schütz does not apply to gastric juice unless this fluid be diluted with fifteen volumes of N/20 hydrochloric acid.

(b) *Fuld and Levison's Method*.—This test is founded upon the fact, shown by Osborne, that edestin when brought into solution in dilute acid will change in its solubility, due to the contact with the acid, and that a protean called *edestan*, which is insoluble in neutral fluid, will be formed. The procedure is as follows: Dilute the gastric juice under examination with 20 volumes of water and introduce gradually decreasing volumes of the diluted juice into a series<sup>4</sup> of narrow test-tubes about 1 cm. in diameter.

<sup>1</sup> In the preparation of these tubes, egg-white is diluted with an equal volume of water, the precipitated globulin filtered off and the filtrate collected in a tall, narrow beaker or a large test-tube. A bundle of capillary tubes about 10 cm. in length and 2 mm. in diameter are now placed in this vessel in such a manner that they are completely submerged in the albumin solution. After an examination has shown that the tubes are completely filled with the albumin solution and that there are no interfering *air-bubbles*, the vessel and its contained tubes is heated for 5-15 minutes in a boiling water-bath, in order to coagulate the albumin. When this coagulation is complete, the tubes are removed, all albumin adhering to them is carefully cleaned off, and the tubes rendered air-tight by the application of sealing wax at either end. When needed for use, these tubes are cut into sections about 2 cm. in length.

<sup>2</sup> Frank: *Journal of Biological Chemistry*, 9, 463, 1911.

<sup>3</sup> Nirenstein and Schiff; *Archiv. für Verdauungs-krankheiten*, 8, 559, 1902.

<sup>4</sup> The longer the series, the more accurate the deductions which may be drawn.



The measurements of gastric juice may conveniently be made with a 1 c.c. pipette which is accurately graduated in 1/100 c.c. Into the first tube in the series may be introduced 1 c.c. of gastric juice, and the tubes which follow in the series may receive volumes which differ, in each instance, from the volume introduced into the preceding tube by 1/100, 1/50, 1/20, or 1/10 of a cubic centimeter. Now *rapidly* introduce into each tube the same volume (*e. g.*, 2 c.c.) of a 1 : 1000 solution of *edestin*<sup>1</sup> and place the tubes at 40° C. for one-half hour. At the end of this time stratify ammonium hydroxide upon the contents of each tube,<sup>2</sup> place the tubes in position before a black background and examine them carefully. The ammonium hydroxide, by diffusing into the acid fluid, forms a neutral zone and in this zone will be precipitated any undigested *edestan* which is present. Select the tube in the series which contains the least amount of gastric juice and which *exhibits no ring*, signifying that the *edestan* has been *completely digested*, and calculate the peptic activity of the gastric juice under examination on the basis of the volume of gastric juice used in this particular tube.

**Calculation.**—Multiply the number of c.c. of edestin solution used by the dilution to which the gastric juice was originally subjected and divide the volume of gastric juice necessary to completely digest the edestan by this product. For example, if 2 c.c. of the edestin solution was completely digested by 0.25 c.c. of a 1 : 20 gastric juice we would have the following expression:  $0.25 \div 20 \times 2$  or 1 : 160. This peptic activity may be expressed in several ways, *e. g.*, (a) 1 : 160 pepsin; (b) 160 pepsin content; (c) 160 parts.

(c) **Rose's Modification<sup>3</sup> of the Jacoby-Solms Method.<sup>4</sup>**—Dissolve 0.25 gram of the globulin of the ordinary garden pea,<sup>5</sup> *Pisum sativum*, in

<sup>1</sup> This edestin should be prepared in the usual way (see p. 109), and brought into solution in a dilute hydrochloric acid of approximately the same strength as that which occurs normally in the human stomach. This may be conveniently made by adding 30 c.c. of N/10 hydrochloric acid to 70 c.c. of water. Ordinarily it should not take longer than *one minute* to introduce the edestin solution into the entire series of tubes. However, if the edestin is added to the tubes in the same order as the ammonium hydroxide is afterward stratified, no appreciable error is introduced.

<sup>2</sup> Making the stratification in the *same order* as the edestin solution was added.

<sup>3</sup> Rose: *Archives of Internal Medicine*, 5, 459, 1910.

<sup>4</sup> Sohns: *Zeitschrift für klinische Medizin*, 64, 159, 1907.

<sup>5</sup> The globulin may be prepared as follows: "The finely ground peas, freed as much as possible from the outer coating, are repeatedly extracted with large quantities of 10 per cent sodium chloride solution, the extracts combined, strained through fine bolting-cloth, and allowed to stand over night in large cylinders to deposit insoluble matter. The supernatant fluid is siphoned off and saturated with ammonium sulphate. The precipitate of albumin and globulin is filtered off, suspended in a little water, and dialyzed in running water for three days, until the salt has been removed, and the albumins have been dissolved. The globulins are filtered off and washed two or three times to remove the last trace of albumins. To purify further, the precipitate is extracted with 10 per cent sodium chloride solution, and filtered until perfectly clear. The resulting solution is neutralized to litmus paper by the cautious addition of dilute sodium hydroxide, and again dialyzed in running water for three days to remove the salts completely. The precipitated globulins are then filtered off and dried on a water-bath at 40° C. During the entire process of separation the proteins should be preserved with a mixture of alcoholic thymol and toluol." This dried globulin is used in the clinical procedure.

100 c.c. of 10 per cent sodium chloride solution, warming slightly if necessary.<sup>1</sup> Filter and introduce 1 c.c. of the clear filtrate into each of a series of six<sup>2</sup> test-tubes about 1 cm. in diameter. Introduce into each tube 1 c.c. of 0.6 per cent hydrochloric acid and permit a period of about five minutes to elapse for the development of the turbidity. Make a known volume of the gastric juice (5-10 c.c. is sufficient) exactly neutral to litmus paper with dilute alkali; and record the volume of the alkali so used. If acid metaprotein precipitates, filter it off; if there is no precipitate proceed without filtration. Dilute the clear neutral solution with a known quantity of distilled water (usually five volumes) making proper allowance for the volume of alkali used in the neutralization. Boil 5-10 c.c. of the diluted juice, filter and add the following decreasing volumes (c.c.) to the series of six tubes: 1.0, 0.9, 0.7, 0.5, 0.2, 0.0. Make the measurements by means of a 1 c.c. pipette graduated in 0.01 c.c. Now rapidly introduce the *unboiled*, diluted juice in the following increasing volumes (c.c.) in order: 0.0, 0.1, 0.3, 0.5, 0.8, 1.0. Each tube now contains a total volume of 3 c.c. and a total acidity of 0.2 per cent hydrochloric acid. Shake each tube thoroughly and place them at 50-52° C. for fifteen minutes or at 35-36° C. for one hour. Examine the series of tubes at the end of the digestion period and select that tube which contains the smallest quantity of gastric juice and which *shows no turbidity*. The volume of the juice used in this tube is taken as the basis for the calculation of the peptic activity.

*Calculation.*—The peptic activity is expressed in terms of 1 c.c. of the *undiluted* juice. For example, if it requires 0.5 c.c. of the diluted juice (five-fold dilution) to clear up the turbidity in 1 c.c. of the globulin solution in the proper experimental time interval (15 minutes or one hour according to temperature) the peptic activity would be expressed as follows:

$$(1 \div 0.5) \times 5 = 10 \text{ (peptic activity).}$$

According to this scale of pepsin units 10 may be considered as "normal" peptic activity. These units are about 1/10 as large as those expressed by the Jacoby-Solms scale.

Inasmuch as it has been shown<sup>3</sup> that blood serum contains an anti-pepsin it is advisable to test the gastric juice for blood before determining its proteolytic power.

**3. Quantitative Determination of Tryptic Activity.**—*Gross' Method.*—This method is based upon the principle that faintly alkaline

<sup>1</sup> This solution may be preserved at least two months under toluol.

<sup>2</sup> A longer series of tubes may be used if desired. However, experience has shown that a series of six ordinarily affords sufficient range for all diagnostic purposes.

<sup>3</sup> Oguro: *Biochemische Zeitschrift*, 22, 266, 1909.

solutions of casein are precipitated upon the addition of dilute (1 per cent) acetic acid whereas its digestion products are not so precipitated. The method follows: Prepare a series of tubes each containing 10 c.c. of a 0.1 per cent solution of pure, fat-free casein,<sup>1</sup> which has been heated to a temperature of 40° C. Add to the contents of the series of tubes increasing amounts of the trypsin solution under examination,<sup>2</sup> and place them at 40° C. for *fifteen minutes*. At the end of this time remove the tubes and acidify the contents of each with a few drops of dilute (1 per cent) acetic acid. The tubes in which the casein is completely digested will remain clear when acidified, while those tubes which contain *undigested* casein will become more or less *turbid* under these conditions. Select the first tube in the series which exhibits *no turbidity* upon acidification, thus indicating complete digestion of the casein, and calculate the tryptic activity of the enzyme solution under examination.

*Calculation.*—The *unit* of tryptic activity is an expression of the power of 1 c.c. of the fluid under examination exerted for a period of fifteen minutes on 10 c.c. of a 0.1 per cent casein solution. For example, if 0.5 c.c. of a trypsin solution completely digests 10 c.c. of a 0.1 per cent solution of casein in fifteen minutes the activity of that solution would be expressed as follows:

$$\text{Tryptic activity} = 1 \div 0.5 = 2.$$

Such a trypsin solution would be said to possess an activity of 2. If 0.3 c.c. of the trypsin solution had been required the solution would be said to possess an activity of 3.3; *i. e.*,  $1 \div 0.3 = 3.3$ .

4. **Quantitative Determination of Catalase.**<sup>3</sup>—In the determination of the catalase values of tissues weighed portions of the tissue under examination should be ground with sand in a mortar then treated with four volumes of chloroform water and permitted to extract for 24 hours at room temperature. An apparatus such as that shown in Fig. 1 may be employed in determining the catalase values. The main features of the apparatus are based upon those of a delivery funnel for introducing liquids under increased or diminished pressure.

In making a determination introduce a measured volume (1–4 c.c.) of the filtered extract<sup>4</sup> into the small flask and insert the modified Johnson burette graduated to 5 c.c. and containing 50 c.c. of hydrogen peroxide (Oakland dioxygen neutral<sup>5</sup> to congo red) into the neck of the flask.

<sup>1</sup> Made by dissolving one gram of Grüber's casein in a liter of 0.1 per cent sodium carbonate. A little chloroform may be added to prevent bacterial action.

<sup>2</sup> The amount of solution used may vary from 0.1–1 c.c. The measurements may conveniently be made by means of a 1 c.c. graduated pipette.

<sup>3</sup> Hawk: *Journal of American Chemical Society*, 33, 425, 1911.

<sup>4</sup> If less than 4 c.c. of extract are used the volume should be made up to 4 c.c. by the addition of distilled water.

<sup>5</sup> An acid reaction modifies the rate of the oxygen evolution. (See Mendel and Leavenworth, *American Journal of Physiology*, 21, 85, 1908.)

Shake the contents of the flask briskly<sup>1</sup> and record the volume of oxygen evolved in a two-minute period taking readings at intervals of fifteen seconds.

*Calculation.*—When a series of comparative tests are made on different tissues or on the same tissue under different conditions it is considered satisfactory to make a comparison of the catalase values upon the basis

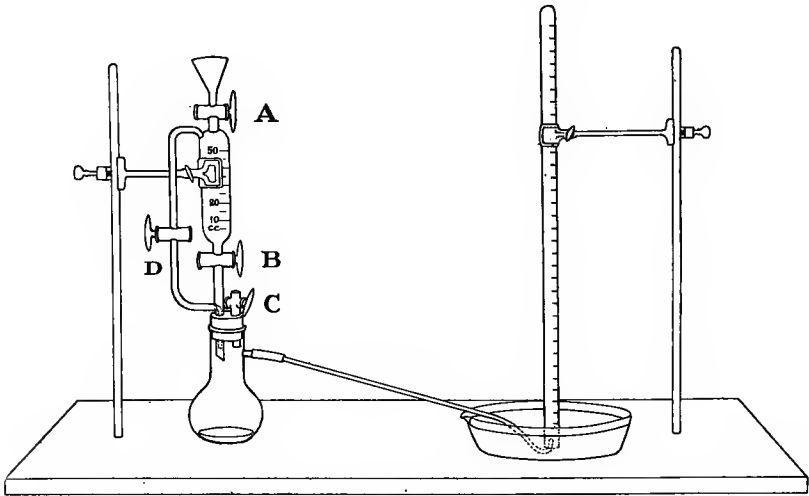


FIG. 1.—APPARATUS FOR QUANTITATIVE DETERMINATION OF CATALASE.

of the *volume of oxygen evolved in a period of two minutes from 5 c.c. of neutral hydrogen peroxide by means of 1 c.c. of a 1:4 chloroform-water extract of the tissue.*

<sup>1</sup> In making a series of comparative tests it is essential that the shaking process should be uniform from determination to determination.

## CHAPTER II.

### CARBOHYDRATES.

The name carbohydrates is given to a class of bodies which are an especially prominent constituent of plants and which are found also in the animal body either free or as an integral part of various proteins. They are called carbohydrates because they contain the elements C, H and O; the H and O being present in the proportion to form water. The term is not strictly appropriate inasmuch as there are bodies, such as acetic acid, lactic acid and inosite, which have H and O present in the proportion to form water, but which are not carbohydrates, and there are also true carbohydrates which do not have H and O present in this proportion, *e. g.*, rhamnose,  $C_6H_{12}O_5$ .

Chemically considered, the carbohydrates are aldehyde or ketone derivatives of complex alcohols. Treated from this standpoint, the aldehyde derivatives are spoken of as aldoses, and the ketone derivatives are spoken of as ketoses. The carbohydrates are also frequently named according to the number of oxygen atoms present in the molecule, *e. g.*, trioses, pentoses, and hexoses.

The more common carbohydrates may be classified as follows:

#### I. Monosaccharides.

##### 1. Hexoses, $C_6H_{12}O_6$ .

- (a) Dextrose.
- (b) Lævulose.
- (c) Galactose.

##### 2. Pentoses, $C_5H_{10}O_5$ .

- (a) Arabinose.
- (b) Xylose.
- (c) Rhamnose (Methyl-pentose),  $C_6H_{12}O_5$ .

#### II. Disaccharides, $C_{12}H_{22}O_{11}$ .

- 1. Maltose.
- 2. Lactose.
- 3. Iso-Maltose.
- 4. Sucrose.

#### III. Trisaccharides, $C_{18}H_{32}O_{16}$ .

- 1. Raffinose.

IV. Polysaccharides,  $(C_6H_{10}O_5)_x$ .

## 1. Starch Group.

- (a) Starch.
- (b) Inulin.
- (c) Glycogen.
- (d) Lichenin.

## 2. Gum and Vegetable Mucilage Group.

- (a) Dextrin.
- (b) Vegetable Gums.

## 3. Cellulose Group.

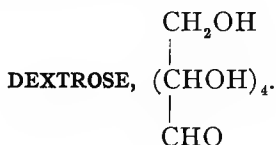
- (a) Cellulose.
- (b) Hemicelluloses.
  - (1) Pentosans.
    - Gum Arabic.
  - (2) Hexosans.
    - Galactans.
    - Agar-agar.

Each member of the above carbohydrate classes, except the members of the pentose group, may be supposed to contain the group  $C_6H_{10}O_5$ , called the *saccharide group*. The polysaccharides consist of this group alone taken a large number of times, whereas the disaccharides may be supposed to contain two such groups plus a molecule of water, and the monosaccharides to contain one such group plus a molecule of water. Thus,  $(C_6H_{10}O_5)_x = \text{polysaccharide}$ ,  $(C_6H_{10}O_5)_2 + H_2O \rightarrow \text{disaccharide}$ ,  $C_6H_{10}O_5 + H_2O \rightarrow \text{monosaccharide}$ . In a general way the solubility of the carbohydrates varies with the number of saccharide groups present, the substances containing the largest number of these groups being the least soluble. This means simply that, as a class, the monosaccharides (hexoses) are the most soluble and the polysaccharides (starches and cellulose) are the least soluble.

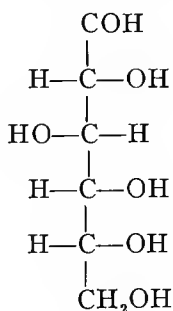
**MONOSACCHARIDES.****Hexoses,  $C_6H_{12}O_6$ .**

The hexoses are monosaccharides containing six oxygen atoms in a molecule. They are the most important of the simple sugars, and two of the principal hexoses, dextrose and lævulose, occur widely distributed in plants and fruits. Of these two hexoses, dextrose results from the hydrolysis of starch whereas both dextrose and lævulose are formed in the hydrolysis of sucrose. Galactose, which with dextrose results from the hydrolysis of lactose, is also an important hexose. These three hexoses

are fermentable by yeast, and yield lævulinic acid upon heating with dilute mineral acids. They reduce metallic oxides in alkaline solution, are optically active and extremely soluble. With phenylhydrazine they form characteristic osazones.



Dextrose, also called glucose or grape sugar, is present in the blood in small amount and may also occur in traces in normal urine. After the ingestion of large amounts of sucrose, lactose or dextrose, causing the *assimilation limit* to be exceeded, an alimentary glycosuria may arise. The assimilation limit for dextrose has been shown<sup>1</sup> to be between 100 and 150 grams. In diabetes mellitus very large amounts of dextrose are excreted in the urine. The following structural formula has been suggested by Victor Meyer for *d*-dextrose:



(For further discussion of dextrose see section on Hexoses, page 26.)

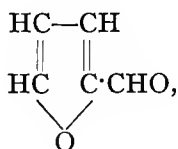
#### EXPERIMENTS ON DEXTROSE.

1. **Solubility.**—Test the solubility of dextrose in the “ordinary solvents” and in alcohol. (In the solubility tests throughout the book we shall designate the following solvents as the “ordinary solvents”: H<sub>2</sub>O; 10 per cent NaCl; 0.5 per cent Na<sub>2</sub>CO<sub>3</sub>; 0.2 per cent HCl; concentrated KOH; concentrated HCl.)

2. **Molisch's Reaction.**—Place approximately 5 c.c. of concentrated H<sub>2</sub>SO<sub>4</sub> in a test-tube. Incline the tube and slowly pour down the inner side of it approximately 5 c.c. of the sugar solution to which 2 drops of Molisch's reagent (a 15 per cent alcoholic solution of  $\alpha$ -naphthol) has been added, so that the sugar solution will not mix with the acid. A reddish-

<sup>1</sup>Brasch: *Zeitschrift für Biologie*, 50, 113, 1907.

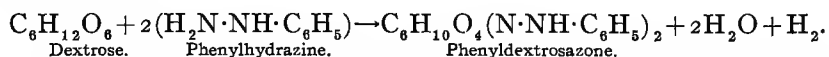
violet zone is produced at the point of contact. The reaction is due to the formation of furfurol,



by the acid. The test is given by all bodies containing a carbohydrate group and is therefore not specific and, in consequence, of very little practical importance.

3. **Phenylhydrazine Reaction.**—Test according to one of the following methods: (a) To a small amount of phenylhydrazine mixture, furnished by the instructor,<sup>1</sup> add 5 c.c. of the sugar solution, shake well and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the tube to cool *slowly* and examine the crystals microscopically (Plate III, opposite). If the solution has become too concentrated in the boiling process it will be light red in color and no crystals will separate until it is diluted with water.

Yellow crystalline bodies called *osazones* are formed from certain sugars under these conditions, in general each individual sugar giving rise to an osazone of a definite crystalline form which is typical for that sugar. It is important to remember in this connection that of the simple sugars of interest in physiological chemistry, dextrose and lævulose yield the same osazone. Each osazone has a definite melting-point and as a further and more accurate means of identification it may be recrystallized and identified by the determination of its melting-point and nitrogen content. The reaction taking place in the formation of *phenyldextrosazone* is as follows:



(b) Place 5 c.c. of the sugar solution in a test-tube, add 1 c.c. of the phenylhydrazine-acetate solution furnished by the instructor,<sup>2</sup> and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the liquid to cool *slowly* and examine the crystals microscopically (Plate III, opposite).

The phenylhydrazine test has been so modified by Cipollina as to be of use as a *rapid clinical test*. The directions for this test are given in the next experiment.

<sup>1</sup>This mixture is prepared by combining one part of phenylhydrazine hydrochloride and two parts of sodium acetate, *by weight*. These are thoroughly mixed in a mortar.

<sup>2</sup>This solution is prepared by mixing one part *by volume*, in each case, of glacial acetic acid, one part of water and two parts of phenylhydrazine (the base).



PLATE III



OSAZONES.

Upper form, dextrosazone; central form, maltosazone; lower form, lactosazone.



4. **Cipollina's Test.**—Thoroughly mix 4 c.c. of dextrose solution, 5 drops of phenylhydrazine (the base) and  $1/2$  c.c. of glacial acetic acid in a test-tube. Heat the mixture for about one minute over a low flame, shaking the tube continually to prevent loss of fluid by bumping. Add 4–5 drops of sodium hydroxide (sp. gr. 1.16), being certain that the fluid in the test-tube remains acid, heat the mixture again for a moment and then cool the contents of the tube. Ordinarily the crystals form at once, especially if the sugar solution possesses a low specific gravity. If they do not appear immediately allow the tube to stand at least 20 minutes before deciding upon the absence of sugar.

Examine the crystals under the microscope and compare them with those shown in Plate III, opposite page 28.

5. **Riegler's Reaction.**<sup>1</sup>—Introduce 0.1 gram of phenylhydrazine-hydrochloride and 0.25 gram of sodium acetate into a test-tube, add 20 drops of the solution under examination and heat the mixture to boiling. Now introduce 10 c.c. of a 3 per cent solution of potassium hydroxide and gently shake the tube and contents. If the solution under examination contains dextrose the liquid in the tube will assume a red color. One per cent dextrose yields an immediate color whereas 0.05 per cent yields the color only after the lapse of a period of one-half hour from the time the alkali is added. In urinary examination if the color appears after the thirty-minute interval the color change is without significance inasmuch as sugar-free urine will respond thus. The reaction is given by all aldehydes and therefore the test cannot be safely employed in testing urines preserved by formaldehyde. Albumin does not interfere with the test.

6. **Bottu's Test.**<sup>2</sup>—To 8 c.c. of Bottu's reagent<sup>3</sup> in a test-tube add 1 c.c. of the solution under examination and mix the liquids by gentle shaking. Now heat the upper portion of the mixture to boiling, add an additional 1 c.c. of the solution and heat the mixture again immediately. The appearance of a blue color accompanied by the precipitation of small particles of indigo blue indicates the present of dextrose in the solution under examination. The test will serve to detect the presence of 0.1 per cent of dextrose.

7. **Precipitation by Alcohol.**—To 10 c.c. of 95 per cent alcohol add about 2 c.c. of dextrose solution. Compare the result with that obtained under Dextrin, 7, page 53.

8. **Iodine Test.**—Make the regular iodine test as given under Starch,

<sup>1</sup>Riegler; Compt. rend. soc. biol., 66, p. 795.

<sup>2</sup>Bottu; Compt. rend. soc. biol., 66, p. 972.

<sup>3</sup>This reagent contains 3.5 grams of *o*-nitrophenylpropionic acid and 5 c.c. of a freshly prepared 10 per cent solution of sodium hydroxide per liter.

5, page 50, and keep this result in mind for comparison with the results obtained later with starch and with dextrin.

9. **Diffusibility of Dextrose.**—Test the diffusibility of dextrose solution through animal membrane, or parchment paper, making a dialyzer like one of the models shown in Fig. 2.

A most satisfactory dialyzing bag may be made of collodion as follows: Pour a solution of collodion into a clean dry Erlenmeyer flask or test-tube. While rotating the vessel on its longitudinal axis, gradually pour out the collodion, at the same time being careful that the interior surface of the flask is completely coated with the solution. Continue the rotation in the inverted position until the collodion ceases to flow. After the solution has evaporated such that the collodion skin on the rim is dry

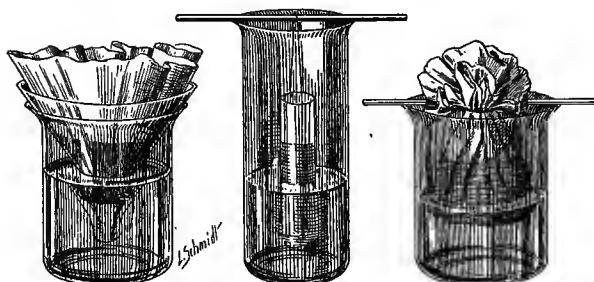


FIG. 2.—DIALYZING APPARATUS FOR STUDENTS' USE.

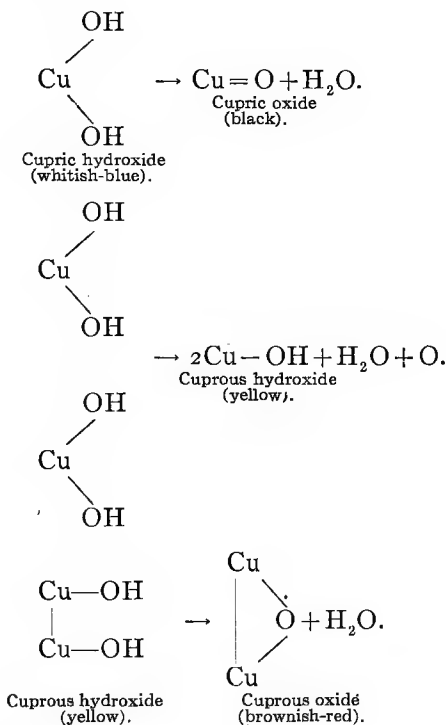
and stiff, cut or loosen it around the edge of the rim. With a pipette or wash bottle run in a few cubic centimeters of water between the membrane and the wall of the flask or test-tube. Shake the inclined vessel while rotating on its longitudinal axis, thus detaching the membrane. Now withdraw the detached bag and fill with water, to determine whether or not it contains defects.<sup>1</sup>

10. **Moore's Test.**—To 2–3 c.c. of sugar solution in a test-tube add an equal volume of concentrated KOH or NaOH, and boil. The solution darkens and finally assumes a brown color. At this point the odor of caramel may be detected. This is an exceedingly crude test and is of little practical value. The brown color is due to the oxidation of the dextrose and the resulting formation of the potassium or sodium salts of certain organic acids which are formed as oxidation products.

11. **Reduction Tests.**—To their aldehyde or ketone structure many sugars owe the property of readily reducing alkaline solutions of the oxides of metals like copper, bismuth and mercury; they also

<sup>1</sup>Gies: Quoted by Clark. *Bioch. Bull.*, 1, 198, 1911

possess the property of reducing ammoniacal silver solutions with the separation of metallic silver. Upon this property of reduction the most widely used tests for sugars are based. When whitish-blue cupric hydroxide in suspension in an alkaline liquid is heated it is converted into insoluble black cupric oxide, but if a reducing agent like certain sugars be present the cupric hydroxide is reduced to insoluble yellow cuprous hydroxide, which in turn, on further heating, may be converted into brownish-red or red cuprous oxide. These changes are indicated as follows:



The chemical equations here discussed are exemplified in Trommer's and Fehling's tests.

(a) *Trommer's Test*.—To 5 c.c. of sugar solution in a test-tube add one-half its volume of KOH or NaOH. Mix thoroughly and add, drop by drop, a *very dilute* solution of copper sulphate. Continue the addition until there is a slight permanent precipitate of cupric hydroxide and in consequence the solution is slightly turbid. Heat, and the cupric hydroxide is reduced to yellow cuprous hydroxide or to brownish-red cuprous oxide. If the solution of copper sulphate used is too strong a small brownish-red precipitate produced in a weak sugar

solution may be entirely masked. On the other hand, particularly in testing for sugar in the urine, if too little copper sulphate is used a light-colored precipitate formed by uric acid and purine bases may obscure the brownish-red precipitate of cuprous oxide. The action of KOH or NaOH in the presence of an excess of sugar and insufficient copper will produce a brownish color. Phosphates of the alkaline earths may also be precipitated in the alkaline solution and be mistaken for cuprous hydroxide. Trommer's test is not very satisfactory.

Salkowski<sup>1</sup> has very recently proposed a modification of the Trommer procedure which he claims is a very accurate sugar test.

(b) *Fehling's Test*.—To about 1 c.c. of Fehling's solution<sup>2</sup> in a test-tube add about 4 c.c. of water, and boil. This is done to determine whether the solution will of itself cause the formation of a precipitate of brownish-red cuprous oxide. If such a precipitate forms, the Fehling's solution must not be used. Add sugar solution to the warm Fehling's solution *a few drops* at a time and heat the mixture after each addition. The production of yellow cuprous hydroxide or brownish-red cuprous oxide indicates that reduction has taken place. The yellow precipitate is more likely to occur if the sugar solution is added rapidly and in large amount, whereas with a less rapid addition of smaller amounts of sugar solution the brownish-red precipitate is generally formed.

This is a much more satisfactory test than Trommer's, but even this test is not entirely reliable when used to detect sugar in the urine. Such bodies as *conjugate glycuronates, uric acid, nucleoprotein* and *homogenistic acid* when present in sufficient amount may produce a result similar to that produced by sugar. *Phosphates of the alkaline earths* may be precipitated by the alkali of the Fehling's solution and in appearance may be mistaken for cuprous hydroxide. Cupric hydroxide may also be reduced to cuprous oxide and this in turn be dissolved by *creatinine*, a normal urinary constituent. This will give the urine under examination a greenish tinge and may obscure the sugar reaction even when a considerable amount of sugar is present.

According to Laird<sup>3</sup> even small amounts of creatinine will *retard the reaction velocity* of reducing sugars with Fehling's solution.

<sup>1</sup> Salkowski; *Zeit. physiol. Chem.*, 79, 164, 1912.

<sup>2</sup> Fehling's solution is composed of two definite solutions—a copper sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

*Copper sulphate solution* = 34.65 grams of copper sulphate dissolved in water and made up to 500 c.c.

*Alkaline tartrate solution* = 125 grams of potassium hydroxide and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

<sup>3</sup> Laird: *Journal of Pathology and Bacteriology*, 16, 398, 1912.

^ (c) *Benedict's Modifications of Fehling's Test.—First Modification.*—To 2 c.c. of Benedict's solution<sup>1</sup> in a test-tube add 6 c.c. of distilled water and 7–9 drops (not more) of the solution under examination. Boil the mixture vigorously for about 15–30 seconds and permit it to cool to room temperature spontaneously. (If desired this process may be repeated, although it is ordinarily unnecessary.) If sugar is present in the solution a precipitate will form which is often *bluish-green* or *green* at first, especially if the percentage of sugar is low, and which usually becomes *yellowish* upon standing. If the sugar present exceeds 0.06 per cent this precipitate generally forms at or below the boiling-point, whereas if less than 0.06 per cent of sugar is present the precipitate forms more slowly and generally only after the solution has cooled.

Benedict claims, whereas the original Fehling test will not serve to detect sugar when present in a concentration of less than 0.1 per cent, that the above modification will serve to detect sugar when present in as small quantity as 0.015–0.02 per cent. Corroboration of this claim of increased delicacy has recently been submitted by Harrison.<sup>2</sup>

The modified Fehling solution used in the above test differs from the original Fehling solution in that 100 grams of sodium carbonate is substituted for the 125 grams of potassium hydroxide ordinarily used, thus forming a Fehling solution which is considerably less alkaline than the original. This alteration in the composition of the Fehling solution is of advantage in the detection of sugar in the urine inasmuch as the strong alkalinity of the ordinary Fehling solution has a tendency, when the reagent is boiled with a urine containing a small amount of dextrose, to decompose sufficient of the sugar to render the detection of the remaining portion exceedingly difficult by the usual technic. Benedict claims that for this reason the use of his modified solution permits the detection of much smaller amounts of sugar than does the use of the ordinary Fehling solution. He has further modified his solution for use in the quantitative determination of sugar (see Chapter XXII).

*Second Modification.*<sup>3</sup>—Very recently Benedict has further modified his solution and has succeeded in obtaining one which does not

<sup>1</sup> Benedict's modified Fehling solution consists of two definite solutions—a copper sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

*Copper sulphate solution* = 34.65 grams of copper sulphate dissolved in water and made up to 500 c.c.

*Alkaline tartrate solution* = 100 grams of anhydrous *sodium carbonate* and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

<sup>2</sup> Harrison: *Pharm. Jour.*, 87, 746, 1911.

<sup>3</sup> Benedict: *Jour. Am. Med. Ass'n.*, 57, 1193, 1911.

deteriorate upon long standing.<sup>1</sup> The following is the procedure for the detection of dextrose in solution: To five cubic centimeters of the reagent in a test-tube add eight (not more) drops of the solution under examination. Boil the mixture vigorously for from one to two minutes and then allow the fluid to cool *spontaneously*. In the presence of dextrose *the entire body of the solution will be filled* with a precipitate, which may be *red, yellow or green* in color, depending upon the amount of sugar present. If no dextrose is present, the solution will remain perfectly clear. (If urine is being tested, it may show a very faint turbidity, due to precipitated urates.) Even very small quantities of dextrose (0.1 per cent) yield precipitates of surprising bulk with this reagent, and the positive reaction for dextrose is the filling of the entire body of the solution with a precipitate, so that the solution becomes opaque. Since *amount* rather than color of the precipitate is made the basis of this test, it may be applied even for the detection of small quantities of dextrose, as readily in artificial light as in daylight.

(d) *Boettger's Test*.—To 5 c.c. of sugar solution in a test-tube add 1 c.c. of KOH or NaOH and a very small amount of bismuth subnitrate, and boil. The solution will gradually darken and finally assume a black color due to reduced bismuth. If the test is made on urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced (bismuth sulphide).

(e) *Nylander's Test (Almén's Test)*.—To 5 c.c. of sugar solution in a test-tube add one-tenth its volume of Nylander's reagent<sup>2</sup> and heat for five minutes in a boiling water-bath.<sup>3</sup> The solution will darken if reducing sugar is present, and upon standing for a few moments a black color will appear. This color is due to the precipitation of bismuth. If the test is made on urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced. Dextrose when present to the

<sup>1</sup> Benedict's new solution has the following composition:

Copper sulphate.....	17.3 grams.
Sodium citrate.....	173.0 grams.
Sodium carbonate (anhydrous).....	100.0 grams.

Distilled water to make 1 liter.

With the aid of heat dissolve the sodium citrate and carbonate in about 600 c.c. of water. Pour (through a folded filter paper if necessary) into a glass graduate and make up to 850 c.c. Dissolve the copper sulphate in about 100 c.c. of water and make up to 150 c.c. Pour the carbonate-citrate solution into a large beaker or casserole and add the copper sulphate solution slowly, with constant stirring. The mixed solution is ready for use and does not deteriorate upon long standing.

<sup>2</sup> Nylander's reagent is prepared by digesting 2 grams of bismuth subnitrate and 4 grams of Rochelle salt in 100 c.c. of a 10 per cent potassium hydroxide solution. The reagent is then cooled and filtered.

<sup>3</sup> Hammarsten suggests that the mixture should be boiled 2-5 minutes (according to the sugar content) over a free flame and the tube then permitted to stand 5 minutes before drawing conclusions.

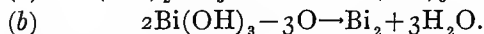


extent of 0.08 per cent may be detected by this reaction. It is claimed by Bechold that Nylander's and Boettger's tests give a negative reaction with solutions containing sugar when mercuric chloride or chloroform is present. Other observers<sup>1</sup> have failed to verify the inhibitory action of mercuric chloride and have shown that the inhibitory influence of chloroform may be overcome by raising the temperature of the urine to the boiling-point for a period of five minutes previous to making the test. Urines rich in *indican*, *urochrome*, *uroerythrin* or *hæmatoporphyrin*, as well as urines excreted after the ingestion of large amounts of certain *medicinal substances*, may give a darkening of Nylander's reagent similar to that of a true sugar reaction. It is a disputed point whether the urine after the administration of urotropin will reduce Nylander's reagent.<sup>2</sup> Strausz<sup>3</sup> has recently shown that the urine of diabetics to whom "Iothion" (diiodohydroxypropane) has been administered will give a negative Nylander's reaction and respond positively to the Fehling and polariscopic tests. "Iothion" also interferes with the Nylander test *in vitro* whereas KI and I do not.

According to Rustin and Otto, the addition of  $\text{PtCl}_4$  increases the delicacy of Nylander's reaction. They claim that this procedure causes the sugar to be converted *quantitatively*. No quantitative method has yet been devised, however, based upon this principle.

Bohmansson<sup>4</sup> before testing the urine under examination treats it (10 c.c.) with 1/5 volume of 25 per cent hydrochloric acid and about 1/2 volume of bone black. This mixture is shaken one minute, then filtered and the neutralized filtrate tested by Nylander's reaction. Bohmansson claims that this procedure removes certain interfering substances, in particular *urochrome*.

A positive Nylander or Boettger test is probably due to the following reactions:



**12. Fermentation Test.**—"Rub up" in a mortar about 20 c.c. of the sugar solution with a small piece of compressed yeast. Transfer the mixture to a saccharometer (shown in Fig. 3, p. 36) and stand it aside in a warm place for about twelve hours. If the sugar is fermentable, alcoholic fermentation will occur and carbon dioxide will collect as a gas in the upper portion of the tube. On the completion of fermenta-

<sup>1</sup> Reh fuss and Hawk; *Journal of Biological Chemistry*, 7, 267, 1910; also Zéidnitz; *Upsala Lakäreforen Forh.*, N. F., 11, 1906.

<sup>2</sup> Abt; *Archives of Pediatrics*, 24, 275, 1907; also Weitbrecht; *Schweiz. Wochschr.*, 47, 577, 1909.

<sup>3</sup> Strausz; *Münch. med. Woch.*, 59, 85, 1912.

<sup>4</sup> Bohmansson; *Biochem. Zeit.*, 19, p. 281.

tion introduce a little potassium hydroxide solution into the graduated portion by means of a bent pipette, place the thumb *tightly* over the opening in the apparatus and invert the saccharometer. Explain the result.

The important findings of Neuberg and associates<sup>1</sup> recently reported indicate very clearly that the liberation of carbon dioxide by yeast is not necessarily a criterion of the presence of sugar. The presence

of a new enzyme called *carboxylase* has been demonstrated in yeast which has the power of *splitting off*  $CO_2$  from the *carboxyl group* of *amino and other aliphatic acids*.

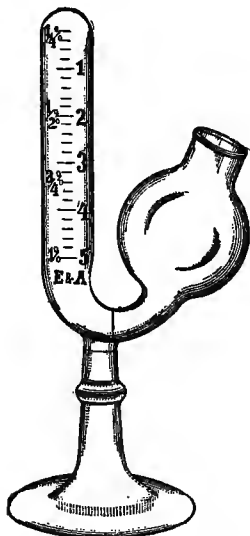


FIG. 3.—EINHORN SACCHAROMETER.

13. **Barfoed's Test.**—Place about 5 c.c. of Barfoed's solution<sup>2</sup> in a test-tube and heat to boiling. Add dextrose solution slowly, a few drops at a time, heating after each addition. Reduction is indicated by the formation of a red precipitate. If the precipitate does not form upon continued boiling allow the tube to stand a few minutes and examine. Sodium chloride interferes with the reaction (Welker).

Barfoed's test is *not* a specific test for dextrose as is frequently stated, but simply serves to detect *monosaccharides*. Disaccharides will also respond to the test, under proper conditions of acidity.<sup>3</sup> Also if the sugar solution is boiled sufficiently long, in contact with the reagent, to hydrolyze the disaccharide through the action of the acetic acid present in the Barfoed's solution a positive test results.<sup>4</sup>

14. **Formation of Caramel.**—Gently heat a small amount of pulverized dextrose in a test-tube. After the sugar has melted and turned brown, allow the tube to cool, add water and warm. The coloring matter produced is known as *caramel*.

15. **Demonstration of Optical Activity.**—A demonstration of the use of the polariscope, by the instructor, each student being required to take readings and compute the "specific rotation."

#### USE OF THE POLARISCOPE.

For a detailed description of the different forms of polariscopes, the method of manipulation and the principles involved, the student is

<sup>1</sup> Neuberg and Associates: *Biochem. Zeitsch.*, 31, 170; 32, 323; 36, (60, 68, 76), 1911.

<sup>2</sup> Barfoed's solution is prepared as follows: Dissolve 4.5 grams of neutral crystallized copper acetate in 100 c.c. of water and add 1.2 c.c. of 50 per cent acetic acid.

<sup>3</sup> Mathews and McGuigan: *Am. Jour. Physiol.*, 19, 175, 1907.

<sup>4</sup> Hinkle and Sherman: *Jour. Am. Chem. Soc.*, 29, 1744, 1907.

referred to any standard text-book of physics. A brief description follows: An ordinary ray of light vibrates in every direction. If such a ray is caused to pass through a "polarizing" Nicol prism it is resolved into *two rays*, one of which vibrates in every direction as before and a second ray which vibrates in *one plane only*. This latter ray is said to be *polarized*. Many organic substances (sugars, proteins, etc.) have the power of twisting or rotating this plane of polarized light, the extent to which the plane is rotated depending upon the number of molecules which

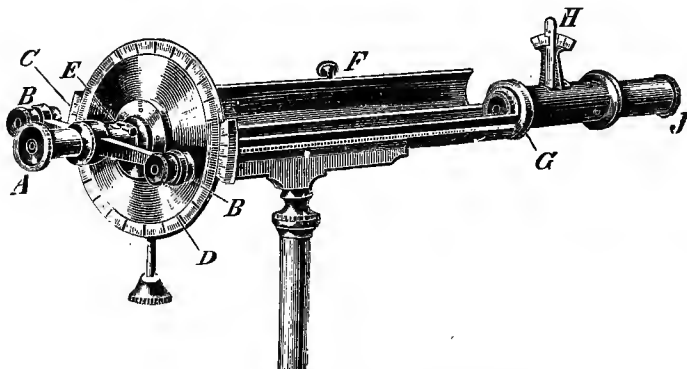


FIG. 4.—ONE FORM OF LAURENT POLARISCOPE.

*B*, Microscope for reading the scale; *C*, a vernier; *E*, position of the analyzing Nicol prism; *H*, polarizing Nicol prism in the tube below this point.

the polarized light passes. Substances which possess this power are said to be "optically active." The *specific rotation* of a substance is the rotation expressed in degrees which is afforded by one gram of substance dissolved in 1 c.c. of water in a tube one decimeter in length. The specific rotation,  $(\alpha)_D$ , may be calculated by means of the following formula,

$$(\alpha)_D = \frac{\alpha}{p \cdot l}$$

in which

$D$  = sodium light.

$\alpha$  = observed rotation in degrees.

$p$  = grams of substance dissolved in 1 c.c. of liquid.

$l$  = length of the tube in decimeters.

If the specific rotation has been determined and it is desired to ascertain the per cent of the substance in solution, this may be obtained by the use of the following formula,

$$p = \frac{\alpha}{(\alpha)_D l}$$

The value of  $p$  multiplied by 100 will be the percentage of the substance in solution.

An instrument by means of which the extent of the rotation may be determined is called a *polariscope* or *polarimeter*. Such an instru-

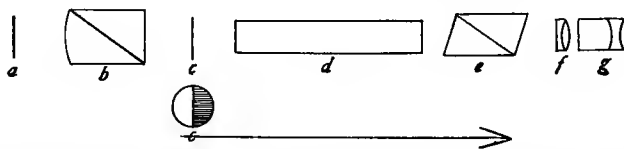


FIG. 5.—DIAGRAMMATIC REPRESENTATION OF THE COURSE OF THE LIGHT THROUGH THE LAURENT POLARISCOPE. (The direction is reversed from that of Fig. 4, p. 37.)

*a*, Bichromate plate to purify the light; *b*, the polarizing Nicol prism; *c*, a thin quartz plate covering one-half the field and essential in producing a second polarized plane; *d*, tube to contain the liquid under examination; *e*, the analyzing Nicol prism; *f* and *g*, ocular lenses.

ment designed especially for the examination of sugar solutions is termed a *saccharimeter* or *polarizing saccharimeter*. The form of polariscope in Fig. 4, p. 37, consists essentially of a long barrel provided with a

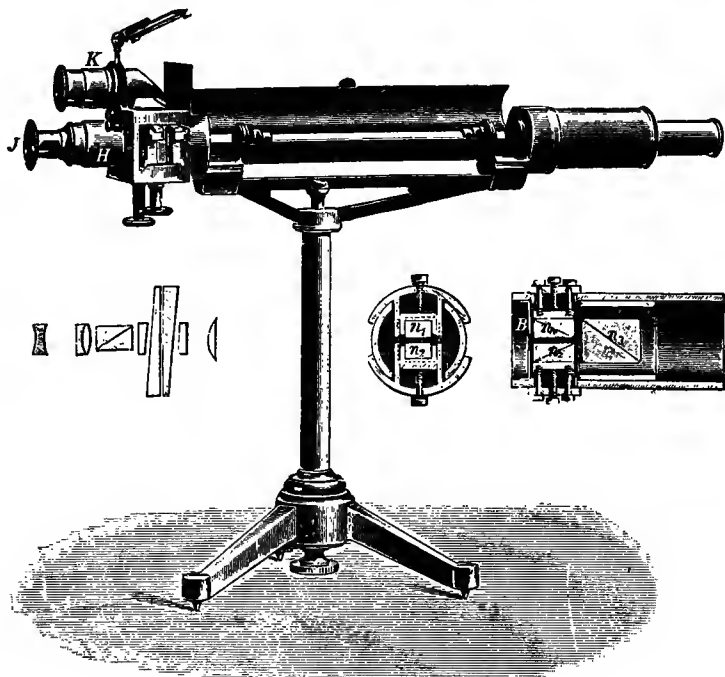


FIG. 6.—POLARISCOPE (SCHMIDT AND HAENSCH MODEL).

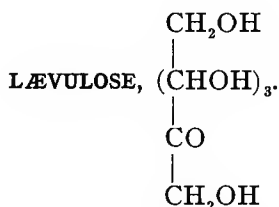
Nicol prism at either end (Fig. 5, above). The solution under examination is contained in a tube which is placed between these two prisms. At the front end of the instrument is an adjusting eyepiece for focusing and

a large recording disc which registers in degrees and fractions of a degree. The light is admitted into the far end of the instrument and is polarized by passing through a Nicol prism. This polarized ray then traverses the column of liquid within the tube mentioned above and if the substance is optically active the plane of the polarized ray is rotated to the right or left. Bodies rotating the ray to the right are called *dextro-rotatory* and those rotating it to the left *laevo-rotatory*.

Within the apparatus is a disc which is so arranged as to be without lines and uniformly light at zero. Upon placing the optically active substance in position, however, the plane of polarized light is rotated or turned and it is necessary to rotate the disc through a certain number of degrees in order to secure the normal conditions, *i. e.*, "without lines and uniformly light." The difference between this reading and the zero is  $\alpha$  or the observed rotation in degrees.

Polarizing saccharimeters are also constructed by which the percentage of sugar in solution is determined by making an observation and multiplying the value of each division on a horizontal sliding scale by the value of the division expressed in terms of dextrose. This factor may vary according to the instrument.

"Optical" methods embracing the determination of the optical activity are being utilized in recent years in many "quantitative" connections.<sup>1</sup>



As already stated, lævulose, sometimes called fructose or fruit sugar, occurs widely disseminated throughout the plant kingdom in company with dextrose. Its reducing power is somewhat weaker than that of dextrose. Lævulose does not ordinarily occur in the urine in diabetes mellitus, but has been found in exceptional cases. With phenylhydrazine it forms the same osazone as dextrose. With methylphenylhydrazine, lævulose forms a characteristic methylphenyllævulosazone.

(For a further discussion of lævulose see the section on Hexoses, p. 26.)

<sup>1</sup> Aberhalden and Schmidt: "Determination of blood content by means of the optical method," *Zeit. physiol. Chem.* 66, 120, 1910; also C. Neuberg; "Determination of nucleic acid cleavage by polarization," *Biochemische Zeitschrift*, 30, 505, 1911

## EXPERIMENTS ON LÆVULOSE.

1-13. Repeat these experiments as given under Dextrose, pages 27-36.

14. **Seliwanoff's Reaction.**—To 5 c.c. of Seliwanoff's reagent<sup>1</sup> in a test-tube add a few drops of a lævulose solution and heat the mixture to boiling. A positive reaction is indicated by the production of a red color and the separation of a red precipitate. The latter may be dissolved in alcohol to which it will impart a striking red color.

If the boiling be prolonged a similar reaction may be obtained with solutions of dextrose or maltose. This has been explained<sup>2</sup> in the case of dextrose as due to the transformation of the dextrose into lævulose by the catalytic action of the hydrochloric acid. The precautions necessary for a positive test for lævulose are as follows: The concentration of the hydrochloric acid must not be more than 12 per cent. The reaction (red color), and the precipitate must be observed after not more than 20-30 seconds boiling. Dextrose must not be present in amounts exceeding 2 per cent. The precipitate must be soluble in alcohol with a bright red color.

15. **Borchardt's Reaction.**—To about 5 c.c. of a solution of lævulose in a test-tube add an equal volume of 25 per cent hydrochloric acid and a few crystals of resorcinol. Heat to boiling and after the production of a red color, cool the tube under running water and transfer to an evaporating dish or beaker. Make the mixture slightly alkaline with *solid* potassium hydroxide, return it to a test-tube, add 2-3 c.c. of acetic ether and shake the tube vigorously. In the presence of lævulose, the acetic ether is colored yellow. (For further discussion of the test see Chapter XIX.)

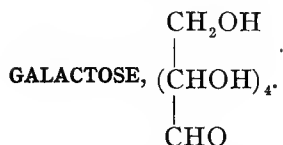
16. **Formation of Methylphenyllævulosazone.**—To a solution of 1.8 grams of lævulose in 10 c.c. of water add 4 grams<sup>3</sup> of methylphenylhydrazine and enough alcohol to clarify the solution. Introduce 4 c.c. of 50 per cent acetic acid and heat the mixture for 5-10 minutes on a boiling water-bath.<sup>4</sup> On standing 15 minutes at room temperature, crystallization begins and is complete in two hours. By scratching the sides of the flask or by inoculation, the solution quickly congeals to form a thick paste of reddish-yellow silky needles. These are the crystals of *methylphenyllævulosazone*. They may be recrystallized from hot 95 per cent alcohol and melt at 153° C.

<sup>1</sup>Seliwanoff's reagent may be prepared by dissolving 0.05 gram of resorcinol in 100 c.c. of dilute (1:2) hydrochloric acid.

<sup>2</sup>Koenigsfeld: *Bioch. Zeit.*, 38, 311, 1912

<sup>3</sup>3.66 grams if absolutely pure.

<sup>4</sup>Longer heating is to be avoided.



Galactose occurs with dextrose as one of the products of the hydrolysis of lactose. It is dextro-rotatory, forms an osazone with phenylhydrazine and ferments slowly with yeast. Upon oxidation with nitric acid galactose yields mucic acid, thus differentiating this monosaccharide from dextrose and l evulose. Lactose also yields mucic acid under these conditions. The mucic acid test may be used in urine examination to differentiate lactose and galactose from other reducing sugars. The assimilation limit for galactose is 30-40 grams.<sup>1</sup>

#### EXPERIMENTS ON GALACTOSE.

1. **Tollens' Reaction.**—To equal volumes of galactose solution and hydrochloric acid (sp. gr. 1.09) add a little phloroglucinol, and heat the mixture on a boiling water-bath. Galactose, pentose and glycuronic acid will be indicated by the appearance of a red color. Galactose may be differentiated from the two latter substances in that its solutions exhibit no absorption bands upon spectroscopical examination.

2. **Mucic Acid Test.**—Treat 100 c.c. of the solution containing galactose with 20 c.c. of concentrated nitric acid (sp. gr. 1.4) and evaporate the mixture in a broad, shallow glass vessel on a boiling water-bath until the volume of the mixture has been reduced to about 20 c.c. At this point the fluid should be *clear*, and a fine white precipitate of *mucic acid* should form. If the percentage of galactose present is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will form. It is impossible to differentiate between galactose and lactose by this test, but the reaction serves to differentiate these two sugars from all other reducing sugars. Differentiate lactose from galactose by means of Barfoed's test (p. 36).

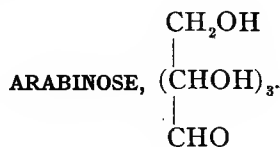
3. **Phenylhydrazine Reaction.**—Make the test according to directions given under Dextrose, 3 or 4, pages 28 and 29.

#### Pentoses, C<sub>5</sub>H<sub>10</sub>O<sub>5</sub>.

In plants and more particularly in certain gums, very complex carbohydrates, called pentosans (see p. 55), occur. These pentosans through hydrolysis by acids may be transformed into pentoses. Pentoses do not ordinarily occur in the animal organism, but have been found in the

<sup>1</sup> Brasch: *Zeitschrift f ur Biologie*, 50, 113, 1907.

urine of morphine habitués and others, their occurrence sometimes being a persistent condition without known cause. They may be obtained from the hydrolysis of nucleoproteins being present in the nucleic acid complex of the molecule. Pentoses are non-fermentable, have strong reducing power and form osazones with phenylhydrazine. Pentoses are an important constituent of the dietary of herbivorous animals. Glycogen is said to be formed after the ingestion of these sugars containing five oxygen atoms. This, however, has not been conclusively proven. On distillation with strong hydrochloric acid pentoses and pentosans yield furfural, which can be detected by its characteristic red reaction with aniline-acetate paper.



Arabinose is one of the most important of the pentoses. The *l*-arabinose may be obtained from gum arabic, plum or cherry gum by boiling for 10 minutes with concentrated hydrochloric acid. This pentose is dextro-rotatory, forms an osazone and has reducing power, but does not ferment. The *d*-arabinose has been isolated from the urine and yields an osazone which melts at 166°–168° C.

#### EXPERIMENTS ON ARABINOSE.

1. **Bial's Reaction.**<sup>1</sup>—To 5 c.c. of Bial's reagent<sup>2</sup> in a test-tube add 2–3 c.c. of the arabinose solution and heat the mixture gently until the first bubbles rise to the surface. Immediately or upon cooling the solution becomes green and a flocculent precipitate of the same color may form. (For further discussion see Chapter XIX). The test may also be performed by adding the pentose to the *hot* reagent.

2. **Tollens' Reaction.**—To equal volumes of arabinose solution and hydrochloric acid (sp. gr. 1.09) add a little phloroglucinol and heat the mixture on a boiling water-bath. Galactose, pentose or glycuronic acid will be indicated by the appearance of a red color. To differentiate between these bodies make a spectroscopic examination and look for the absorption band between *D* and *E* given by pentoses and glycuronic acid. Differentiate between the two latter bodies by the melting-points of their osazones.

<sup>1</sup> Bial: *Deut. med. Woch.*, 28, 252, 1902.

<sup>2</sup> Orcinol.....1.5 gram.

Fuming HCl.....500 grams.

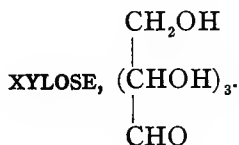
Ferric chloride (10 per cent) ..20–30 drops.



Compare the reaction with that obtained with galactose (page 41).

3. **Orcinol Test.**—Repeat 1, using orcinol instead of phloroglucinol. A succession of colors from red through reddish-blue to green is produced. A green precipitate is formed which is soluble in amyl alcohol and has absorption bands between *C* and *D*.

4. **Phenylhydrazine Reaction.**—Make this test on the arabinose solution according to directions given under Dextrose, 3 or 4, pages 28 and 29.



Xylose, or wood sugar, is obtained by boiling wood gums with dilute acids as explained under Arabinose, page 43. It is dextro-rotatory, forms an osazone and has reducing power, but does not ferment.

#### EXPERIMENTS ON XYLOSE.

1-4. Same as for arabinose (see above).

#### RHAMNOSE, $\text{C}_6\text{H}_{12}\text{O}_5$ .

Rhamnose or methyl-pentose is an example of a true carbohydrate which does not have the H and O atoms present in the proportion to form water. Its formula is  $\text{C}_6\text{H}_{12}\text{O}_5$ . It has been found that rhamnose when ingested by rabbits or hens has a positive influence upon the formation of glycogen in those organisms.

#### DISACCHARIDES, $\text{C}_{12}\text{H}_{22}\text{O}_{11}$ .

The disaccharides as a class may be divided into two rather distinct groups. The first group would include those disaccharides which are found in nature as such, *e. g.*, *sucrose* and *lactose* and the second group would include those disaccharides formed in the hydrolysis of more complex carbohydrates, *e. g.*, *maltose*, and *iso-maltose*.

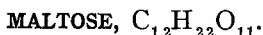
The disaccharides have the general formula  $\text{C}_{12}\text{H}_{22}\text{O}_{11}$ , to which, in the process of hydrolysis, a molecule of water is added causing the single disaccharide molecule to split into two monosaccharide (hexose) molecules. The products of the hydrolysis of the more common disaccharides are as follows:

Maltose = dextrose + dextrose.

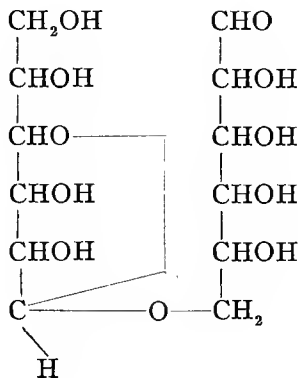
Lactose = dextrose + galactose.

Sucrose = dextrose + lævulose.

All of the more common disaccharides *except sucrose* have the power of reducing certain metallic oxides in alkaline solution, notably those of copper and bismuth. This reducing power is due to the presence of the *aldehyde group* ( $-\text{CHO}$ ) in the sugar molecule.

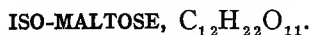


Maltose or malt sugar is formed in the hydrolysis of starch through the action of an enzyme, *vegetable amylase (diastase)*, contained in sprouting barley or malt. Certain enzymes in the saliva and in the pancreatic juice may also cause a similar hydrolysis. Maltose is also an intermediate product of the action of dilute mineral acids upon starch. It is strongly dextro-rotatory, reduces metallic oxides in alkaline solution and is fermentable by yeast after being inverted (see Polysaccharides, page 47) by the enzyme *maltase* of the yeast. In common with the other disaccharides, maltose may be hydrolyzed with the formation of two molecules of monosaccharide. In this instance the products are two molecules of dextrose. With phenylhydrazine maltose forms an osazone, *maltosazone*. The following formula represents the probable structure of maltose:



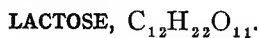
#### EXPERIMENTS ON MALTOSE.

1-13. Repeat these experiments as given under Dextrose, pages 27-36.



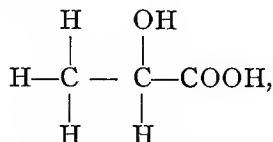
Iso-maltose, an isomeric form of maltose, is formed, along with maltose, by the action of diastase upon starch paste, and also by the action of hydrochloric acid upon dextrose. It also occurs with maltose as one of the products of salivary digestion. It is dextro-rotatory and with phenylhydrazine gives an osazone which is characteristic. Iso-maltose is very

soluble and reduces the oxides of bismuth and copper in alkaline solution. Pure iso-maltose is probably only slightly fermentable.



Lactose or milk sugar occurs ordinarily only in milk, but has often been found in the urine of women during pregnancy and lactation. It may also occur in the urine of normal persons after the ingestion of unusually large amounts of lactose in the food. It has a strong reducing power, is dextro-rotatory and forms an osazone with phenylhydrazine. Upon hydrolysis lactose yields one molecule of dextrose and one molecule of galactose.

In the souring of milk the bacterium *lactis* and certain other micro-organisms bring about lactic acid fermentation by transforming the lactose of the milk into lactic acid,



and alcohol. This same reaction may occur in the alimentary canal as the result of the action of putrefactive bacteria. In the preparation of kephyr and koumyss the lactose of the milk undergoes alcoholic fermentation, through the action of ferments other than yeast, and at the same time lactic acid is produced. Lactose and galactose yield *mucic acid* on oxidation with nitric acid. This fact is made use of in urine analysis to facilitate the differentiation of these sugars from other reducing sugars.

Lactose is *not* fermentable by pure yeast.

#### EXPERIMENTS ON LACTOSE.

1-13. Repeat these experiments as given under Dextrose, pages 27-36.

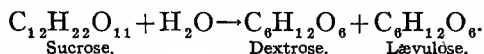
14. **Mucic Acid Test.**—Treat 100 c.c. of the solution containing lactose with 20 c.c. of concentrated nitric acid (sp. gr. 1.4) and evaporate the mixture in a broad, shallow glass vessel on a boiling water-bath, until the volume of the mixture has been reduced to about 20 c.c. At this point the fluid should be *clear*, and a fine white precipitate of *mucic acid* should form. If the percentage of lactose present is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will appear. It is impossible to differentiate between lactose and galactose by this test, but the reaction serves to differentiate these two sugars from all other reducing sugars.

Differentiate lactose from galactose by means of Barfoed's test, page 36.

### SUCROSE, $C_{12}H_{22}O_{11}$ .

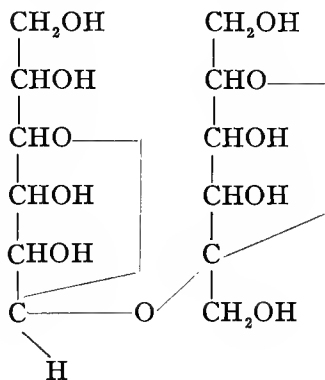
Sucrose, also called saccharose or cane sugar, is one of the most important of the sugars and occurs very extensively distributed in plants, particularly in the sugar cane, sugar beet, sugar millet and in certain palms and maples.

Sucrose is dextro-rotatory and upon hydrolysis, as before mentioned, the molecule of sucrose takes on a molecule of water and breaks down into two molecules of monosaccharide. The monosaccharides formed in this instance are dextrose and lævulose. This is the reaction:



This process is called *inversion* and may be produced by bacteria, enzymes, and certain weak acids. After this inversion the previously strongly dextro-rotatory solution becomes lævo-rotatory. This is due to the fact that the lævulose molecule is more strongly lævo-rotatory than the dextrose molecule is dextro-rotatory. The product of this inversion is called *invert sugar*.

Sucrose does *not* reduce metallic oxides in alkaline solution and forms *no* osazone with phenylhydrazine. It is not fermentable directly by yeast, but must first be *inverted* by the enzyme *sucrase* (*invertase* or *invertin*) contained in the yeast. The probable structure of sucrose may be represented by the following formula. Note the absence of any free ketone or aldehyde group.



### EXPERIMENTS ON SUCROSE.

1-13. Repeat these experiments according to the directions given under Dextrose, pages 27-36.

14. **Inversion of Sucrose.**—To 25 c.c. of sucrose solution in a beaker add 5 drops of concentrated HCl and boil one minute. Cool the solution, render alkaline with *solid* KOH and upon the resulting fluid repeat experiments 3 (or 4) and 11 as given under Dextrose, pages 28–30. Explain the results.

15. **Production of Alcohol by Fermentation.**—Prepare a strong (10–20 per cent) solution of sucrose, add a small amount of egg albumin or commercial peptone and introduce the mixture into a bottle of appropriate size. Add yeast, and by means of a bent tube inserted through a stopper into the neck of the bottle, conduct the escaping gas into water. As fermentation proceeds readily in a warm place the escaping gas may be collected in a eudiometer tube and examined. When the activity of the yeast has practically ceased, filter the contents of the bottle into a flask and distil the mixture. Catch the first portion of the distillate separately and test for alcohol by one of the following reactions:

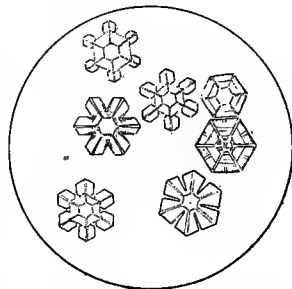


FIG. 7.—IODOFORM. (*Autenrieth.*)

(a) *Iodoform Test.*—Render 2–3 c.c. of the distillate alkaline with potassium hydroxide solution and add a few drops of iodine solution. Heat gently and note the formation of iodoform crystals. Examine these crystals under the microscope and compare them with those in Fig. 7.

(b) *Aldehyde Test.*—Place 5 c.c. of the distillate in a test-tube, add a few drops of potassium dichromate solution,  $K_2Cr_2O_7$ , and render it acid with dilute sulphuric acid. Boil the acid solution and note the odor of aldehyde.

## TRISACCHARIDES, $C_{18}H_{32}O_{16}$ .

### RAFFINOSE.

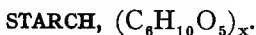
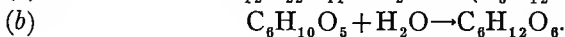
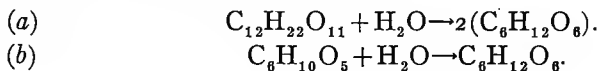
This trisaccharide, also called melitose, or melitriose occurs in cotton seed, Australian manna, and in the molasses from the preparation of beet sugar. It is dextro-rotatory, does not reduce Fehling's solution, and is only partly fermentable by yeast.

Raffinose may be hydrolyzed by weak acids the same as the polysaccharides are hydrolyzed, the products being lævulose and melibiose; further hydrolysis of the melibiose yields dextrose and galactose.

## POLYSACCHARIDES, $(C_6H_{10}O_5)_x$ .

In general the polysaccharides are amorphous bodies, a few, however, are crystallizable. Through the action of certain enzymes or

weak acids the polysaccharides may be hydrolyzed with the formation of monosaccharides. As a class the polysaccharides are quite insoluble and are non-fermentable until inverted. By inversion is meant the hydrolysis of disaccharide or polysaccharide sugars to form monosaccharides, as indicated in the following equations:



Starch is widely distributed throughout the vegetable kingdom, occurring in grains, fruits, and tubers. It occurs in granular form, the microscopical appearance being typical for each individual starch. The granules, which differ in size according to the source, are composed of alternating concentric rings of granulose and cellulose. Ordinary starch is insoluble in cold water, but if boiled with water the cell walls are ruptured and *starch paste* results. In general starch gives a *blue* color with iodine.

Starch is acted upon by amylases, *e. g.*, salivary amylase (*ptyalin*) and pancreatic amylase (*amylopsin*), with the formation of *soluble starch*, *erythro-dextrin*, *achroo-dextrins*, *maltose*, *iso-maltose* and *dextrose* (see Salivary Digestion, page 61). Maltose is the principal end-product of this enzyme action. Upon boiling a starch solution with a dilute mineral acid a series of similar bodies is formed, but under these conditions *dextrose* is the principal end-product.

#### EXPERIMENTS ON STARCH.

1. **Preparation of Potato Starch.**—Pare a raw potato, comminute it upon a fine grater, mix with water, and “whip up” the pulped material vigorously before straining it through cheese cloth or gauze to remove the coarse particles. The starch rapidly settles to the bottom and can be washed by repeated decantation. Allow the compact mass of starch to drain thoroughly and spread it out on a watch glass to dry in the air. If so desired this preparation may be used in the experiments which follow.

2. **Microscopical Examination.**—Examine microscopically the granules of the various starches submitted and compare them with those shown in Figs. 8–18, page 49. The suspension of the granules in a drop of water will facilitate the microscopical examination.

3. **Solubility.**—Try the solubility of one form of starch in each of the ordinary solvents (see page 27). If uncertain regarding the solubility in any reagent, filter and test the filtrate with iodine solution as given

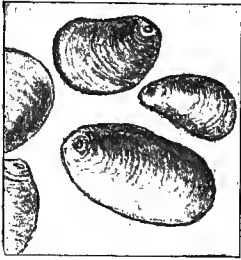


FIG. 8.—POTATO.

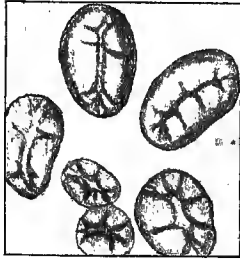


FIG. 9.—BEAN.



FIG. 10.—ARROWROOT.

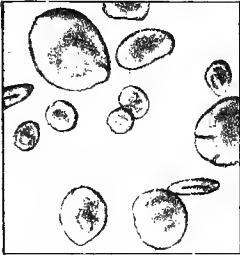


FIG. 11.—RYE.

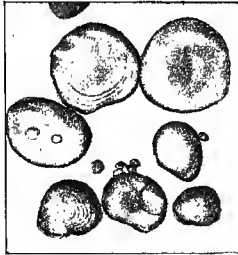


FIG. 12.—BARLEY.

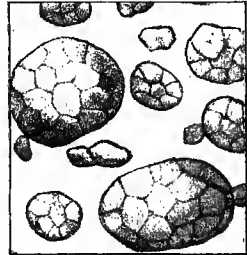


FIG. 13.—OAT.



FIG. 14.—BUCKWHEAT.



FIG. 15.—MAIZE.



FIG. 16.—RICE.



FIG. 17.—PEA.



FIG. 18.—WHEAT.

STARCH GRANULES FROM VARIOUS SOURCES. (*Lefmarn and Beam*)

under 5 below. The production of a blue color would indicate that the starch had been dissolved by the solvent.

4. **Iodine Test.**—Place a few granules of starch in one of the depressions of a porcelain test-tablet and treat with a drop of a dilute solution of iodine in potassium iodide. The granules are colored blue due to the formation of so-called *iodide of starch*. The cellulose of the granule is not stained as may be seen by examining microscopically.

5. **Iodine Test on Starch Paste.**<sup>1</sup>—Repeat the iodine test using the starch paste. Place 2–3 c.c. of starch paste<sup>2</sup> in a test-tube, add a drop of the dilute iodine solution and observe the production of a blue color. Heat the tube and note the disappearance of the color. It reappears on cooling.

In similar tests note the influence of alcohol and of alkali upon the so-called iodide of starch.

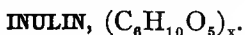
The composition of the iodide of starch is not definitely known.

6. **Fehling's Test.**—On starch paste (see page 32).

7. **Hydrolysis of Starch.**—Place about 25 c.c. of starch paste in a small beaker, add 10 drops of concentrated HCl, and boil. By means of a small pipette, at the end of each minute, remove a drop of the solution to the test-tablet and make the regular iodine test. As the testing proceeds the blue color should gradually fade and finally disappear. At this point, after cooling and neutralizing with solid KOH, Fehling's test (see page 32) should give a positive result due to the formation of a reducing sugar from the starch. Make the phenylhydrazine test upon some of the hydrolyzed starch. What sugar has been formed?

8. **Influence of Tannic Acid.**—Add an excess of tannic acid solution to a small amount of starch paste in a test-tube. The liquid will become strongly opaque and ordinarily a yellowish-white precipitate is produced. Compare this result with the result of the similar experiment on dextrin (page 53).

9. **Diffusibility of Starch Paste.**—Test the diffusibility of starch paste through animal membrane, parchment paper or collodion, making a dialyzer like one of the models shown in Fig. 2, page 30.



Inulin is a polysaccharide which may be obtained as a white, odorless, tasteless powder from the tubers of the artichoke, elecampane, or dahlia.

<sup>1</sup> *Preparation of Starch Paste.*—Grind 2 grains of starch powder in a mortar with a small amount of cold water. Bring 200 c.c. of water to the boiling-point and add the starch mixture from the mortar with continuous stirring. Bring again to the boiling-point and allow it to cool. This makes an approximate 1 per cent starch paste which is a very satisfactory strength for general use.

<sup>2</sup> For this particular test a starch paste of very satisfactory strength may be made by mixing 1 c.c. of a 1 per cent starch paste with 100 c.c. of water.



It has also been prepared from the roots of chicory, dandelion, and burdock. It is very slightly soluble in cold water and quite easily soluble in hot water. In cold alcohol of 60 per cent or over it is practically insoluble. Inulin gives a negative reaction with iodine solution. The "yellow" color reaction with iodine mentioned in many books is doubtless merely the normal color of the iodine solution. It is very difficult to prepare inulin which does not reduce Fehling's solution slightly. This reducing power may be due to an impurity. Practically all commercial preparations of inulin possess considerable reducing power.

Inulin is lævo-rotatory and upon hydrolysis by acids or by the enzyme *inulase* it yields the monosaccharide lævulose which readily reduces Fehling's solution. The ordinary amylolytic enzymes occurring in the animal body do not digest inulin. A small part of the ingested inulin may be hydrolyzed by the acid gastric juice, but Lewis<sup>1</sup> has recently shown that "the value of inulin as a significant source of energy in human dietaries must be questioned."

#### EXPERIMENTS ON INULIN.

1. **Solubility.**—Try the solubility of inulin powder in each of the ordinary solvents. If uncertain regarding the solubility in any reagent, filter and neutralize the filtrate if it is alkaline in reaction. Add a drop of concentrated hydrochloric acid to the filtrate and boil it for one minute. Render the solution neutral or slightly alkaline with *solid* potassium hydroxide and try Fehling's test. What is the significance of a positive Fehling's test in this connection?

2. **Iodine Test.**—(a) Place 2–3 c.c. of the inulin solution in a test-tube and add a drop of dilute iodine solution. What do you observe?

(b) Place a small amount of inulin powder in one of the depressions of a test-tablet and add a drop of dilute iodine solution. Is the effect any different from that observed above?

3. **Molisch's Reaction.**—Repeat this test according to directions given under Dextrose, 2, page 27.

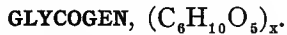
4. **Fehling's Test.**—Make this test on the inulin solution according to the instructions given under Dextrose, page 32. Is there any reduction?<sup>2</sup>

5. **Hydrolysis of Inulin.**—Place 5 c.c. of inulin solution in a test-tube, add a drop of concentrated hydrochloric acid and boil it for one minute. Now cool the solution, neutralize it with concentrated KOH and test the

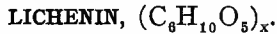
<sup>1</sup> Lewis: *Journal American Medical Ass'n.*, 58, 1176, 1912.

<sup>2</sup> See the discussion of the properties of inulin, above.

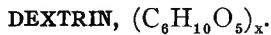
reducing action of 1 c.c. of the solution upon 1 c.c. of diluted (1:4) Fehling's solution. Explain the result.<sup>1</sup>



(For discussion and experiments see Muscular Tissue, Chapter XV.)



Lichenin may be obtained from *Cetraria islandica* (Iceland moss). It forms a difficultly soluble jelly in cold water and an opalescent solution in hot water. It is optically inactive and gives no color with iodine. Upon hydrolysis with dilute mineral acids lichenin yields dextrans and dextrose. It is said to be most nearly related chemically to starch. Saliva, pancreatic juice, malt diastase and gastric juice have no noticeable action on lichenin.



The dextrans are the bodies formed midway in the stages of the hydrolysis of starch by weak acids or an enzyme. They are amorphous bodies which are easily soluble in water, acids, and alkalis, but are insoluble in alcohol or ether. Dextrans are dextro-rotatory and are not fermentable by yeast.

The dextrans may be hydrolyzed by dilute acids to form dextrose. With iodine one form of dextrin (erythro-dextrin) gives a red color. Their power to reduce Fehling's solution is questioned.

#### EXPERIMENTS ON DEXTRIN.

1. **Solubility.**—Test the solubility of pulverized dextrin in the ordinary solvents (see page 27).

2. **Iodine Test.**—Place a drop of dextrin solution in one of the depressions of the test-tablet and add a drop of a dilute solution of iodine in potassium iodide. A red color results due to the formation of the *red iodide of dextrin*. If the reaction is not sufficiently pronounced make a stronger solution from pulverized dextrin and repeat the test. The solution should be slightly acid to secure the best results.

Make proper tests to show that the *red iodide of dextrin* is influenced by heat, alkali, and alcohol in a similar manner to the *blue iodide of starch* (see page 50).

<sup>1</sup> If the inulin solution gave a positive Fehling test in the last experiment it will be necessary to check the hydrolysis experiment as follows: To 5 c.c. of the inulin solution in a test-tube add one drop of concentrated hydrochloric acid, neutralize with concentrated KOH solution and test the reducing action of 1 c.c. of the resulting solution upon 1 c.c. of diluted (1:4) Fehling's solution. This will show the normal reducing power of the inulin solution. In case the inulin was hydrolyzed, the Fehling's test in the hydrolysis experiment should show a more pronounced reduction than that observed in the check experiment.

3. **Fehling's Test.**—See if the dextrin solution will reduce Fehling's solution.

4. **Hydrolysis of Dextrin.**—Take 25 c.c. of dextrin solution in a small beaker, add 5 drops of dilute hydrochloric acid, and boil. By means of a small pipette, at the end of each minute, remove a drop of the solution to one of the depressions of the test-tablet and make the iodine test. The power of the solution to produce a color with iodine should rapidly disappear. When a negative reaction is obtained cool the solution and neutralize it with concentrated potassium hydroxide. Try Fehling's test (see page 32). This reaction is now strongly positive, due to the formation of a reducing sugar. Determine the nature of the sugar by means of the phenylhydrazine test (see pages 28 and 29).

5. **Influence of Tannic Acid.**—Add an excess of tannic acid solution to a small amount of dextrin solution in a test-tube. No precipitate forms. This result differs from the result of the similar experiment upon starch (see Starch, 8, page 50).

6. **Diffusibility of Dextrin.**—(See Starch, 9, page 50.)

7. **Precipitation by Alcohol.**—To about 50 c.c. of 95 per cent alcohol in a small beaker add about 10 c.c. of a *concentrated* dextrin solution. Dextrin is thrown out of solution as a gummy white precipitate. Compare the result with that obtained under Dextrose, 5, page 50.

### CELLULOSE, $(C_6H_{10}O_5)_x$

This complex polysaccharide forms a large portion of the cell wall of plants. It is extremely insoluble and its molecule is much more complex than the starch molecule. The best quality of filter paper and the ordinary absorbent cotton are good types of cellulose.

At one time there was but a single known solvent for cellulose. Recent investigation has, however, revealed a long list of cellulose solvents. (See Experiment 7.)

Cellulose is not hydrolyzed by boiling with dilute mineral acids. It may be hydrolyzed, however, by treating with concentrated sulphuric acid then subsequently diluting the solution with water and boiling.

There is some difference of opinion as to the exact extent to which cellulose is utilized in the animal organism. It is no doubt, more efficiently utilized by herbivora than by carnivora or by man. It is claimed that about 25 per cent may be utilized by herbivora, less than 5 per cent by dogs whereas the quantity utilized by man is "too small for it to play a rôle of importance in the diet of a normal individual."<sup>1</sup> In neither man nor the lower animals has there been demonstrated any formation of

<sup>1</sup>Swartz: Transactions of the Connecticut Academy of Arts and Sciences, 16, 247, 1911.

sugar or glycogen from cellulose.<sup>1</sup> It is probable that the cellulose which disappears from the intestine is transformed for the most part into fatty acids.<sup>2</sup>

### EXPERIMENTS ON CELLULOSE.

1. **Solubility.**—Test the solubility of cellulose in the ordinary solvents (see page 27).

2. **Iodine Test.**—Add a drop of dilute iodine solution to a few shreds of cotton on a test-tablet. Cellulose differs from starch and dextrin in giving *no color* with iodine.

3. **Formation of Amyloid.**<sup>3</sup>—Add 10 c.c. of dilute and 5 c.c. of concentrated  $H_2SO_4$  to some absorbent cotton in a test-tube. When entirely dissolved (without heating) pour one-half of the solution into another test-tube, cool it and dilute with water. Amyloid forms as a gummy precipitate and gives a brown or blue coloration with iodine.

After allowing the second portion of the acid solution of cotton to stand about 10 minutes, dilute it with water in a small beaker and boil for 15–30 minutes. Now cool, neutralize with *solid* KOH and test with Fehling's solution. Dextrose has been formed from the cellulose by the action of the acid.

4. **Schweitzer's Solubility Test.**—Place a little absorbent cotton in a test-tube, add Schweitzer's reagent,<sup>4</sup> and stir the cellulose with a glass rod. When completely dissolved acidify the solution with acetic acid. An amorphous precipitate of cellulose is produced.

5. **Cross and Bevan's Solubility Test.**<sup>5</sup>—Place a little absorbent cotton in a test-tube, add Cross and Bevan's reagent,<sup>6</sup> and stir the cellulose with a glass rod. When solution is complete reprecipitate the cellulose with 95 per cent alcohol.

6. **Iodine-Zinc Chloride Reaction.**—Place a little absorbent cotton or quantitative filter paper in a test-tube and treat it with the iodine-zinc chloride reagent.<sup>7</sup> A *blue* color forms on standing. Amyloid has been

<sup>1</sup> Lusk: *American Journal of Physiology*, 27, 467, 1911; also Hoffmann, Inaugural dissertation, Halle-Wittenberg, 1910.

<sup>2</sup> Tappeiner: *Zeitschrift für Biologie*, 24, 105, 1888.

<sup>3</sup> This body derives its name from *amylum* (starch) and is not to be confounded with amyloid, the glycoprotein.

<sup>4</sup> Schweitzer's reagent is made by adding potassium hydroxide to a solution of copper sulphate which contains some ammonium chloride. A precipitate of cupric hydroxide forms and this is filtered off, washed, and 3 grams of the moist cupric hydroxide brought into solution in a liter of 20 per cent ammonium hydroxide.

<sup>5</sup> Cross and Bevan: *Chemical News*, 63, p. 66.

<sup>6</sup> Cross and Bevan's reagent may be prepared by combining two parts of concentrated hydrochloric acid and one part of zinc chloride, *by weight*.

<sup>7</sup> The iodine-zinc chloride reagent as suggested by Nowopokrowsky (*Beihfte Botan. Centr.*, 28, 90, 1912) may be made by dissolving 20 grams  $ZnCl_2$  in 8.5 c.c. water and when cool introducing the iodine solution (3 grams KI + 1.5 gram I in 60 c.c. water) drop by drop until iodine begins to precipitate.

formed from the cellulose through the action of the  $ZnCl_2$  and the iodine solution has stained the amyloid blue.

**7. New Cellulose Solvents.**—It has recently been demonstrated by Deming<sup>1</sup> that there are many excellent solvents for cellulose (filter paper). For example, the concentrated aqueous solutions of certain salts such as *antimony trichloride*, *stannous chloride* and *zinc bromide*. In hydrochloric acid solution the solvent action of the above salts is increased. The following salts are also good solvents in hydrochloric acid solution: *mercuric chloride*, *bismuth chloride*, *antimony pentachloride*, *tin tetrachloride* and *titanium tetrachloride*. In the case of the last-mentioned salt the swollen, transparent character of the cellulose fibers preliminary to solution can be seen very nicely.

Try selected solvents suggested by the instructor.

#### HEMICELLULOSES.

The hemicelluloses differ from cellulose in that they may be hydrolysed upon boiling with dilute mineral acids. They differ from other polysaccharides in not being readily digested by amylases. Hemicellulose may yield pentosans, or hexosans upon hydrolysis.

*Pentosans.*—Pentosans yield pentoses upon hydrolysis. So far as is known they do not occur in the animal kingdom. They have, however, a very wide distribution in the vegetable kingdom, being present in leaves, roots, seeds and stems of all forms of plants, many times in intimate association or even chemical combination with galactans. In herbivora, pentosans are 40–80 per cent utilized.<sup>2</sup> The few tests on record as to the pentosan utilization by man<sup>3</sup> indicate that 80–95 per cent disappear from the intestine. According to Cramer,<sup>4</sup> bacteria are efficient hemicellulose transformers. It has not yet been demonstrated that pentosans form glycogen in man, and for this reason they must be considered as playing an unimportant part in human nutrition. Gum arabic an important pentosan may be hydrolyzed by concentrated hydrochloric acid if boiled for a short time. The pentose arabinose results from such hydrolysis.

*Galactans.*—In common with the pentosans the galactans have a very wide distribution in the vegetable kingdom. The pure galactans yield galactose upon hydrolysis. One of the most important members of the galactan group is *agar-agar*, a product prepared from certain types of Asiatic sea-weed. This galactan is about 50 per cent utilizable by herbivora<sup>5</sup> and 8–27 per cent utilizable by man.<sup>6</sup> Agar ingestion has

<sup>1</sup> Deming: *Journal American Chemical Society*, 33, 1515, 1911.

<sup>2</sup> Swartz: Transactions of the Connecticut Academy of Arts and Sciences, 16, 247, 1911.

<sup>3</sup> König and Reinhardt: *Zeit. f. Untersuchung der Nahrungs u. Genussmittel*, 5, 110, 1902.

<sup>4</sup> Cramer: Inaug. Diss., Halle, 1910.

<sup>5</sup> Lohrisch: *Zeit. f. exper. Path. u. Pharm.*, 5, 478, 1908.

<sup>6</sup> Saiki: *Jour. Biol. Chem.*, 2, 251, 1906.

been shown to be a very efficient therapeutic aid in cases of chronic constipation.<sup>1, 2</sup> This is particularly true when the constipation is due to the formation of dry, hard, fecal masses (*scybala*), a type of fecal formation which frequently follows the ingestion of a diet which is very thoroughly digested and absorbed. The agar, because of its relative indigestibility and its property of absorbing water yields a bulky fecal mass which is sufficiently soft to permit of easy evacuation. Agar has been used with good results in the treatment of constipation in children.<sup>3</sup> The function of agar is not limited to its use in connection with constipation, it may serve in other capacities as an aid to intestinal therapeutics.<sup>4</sup>

#### EXPERIMENTS ON A PENTOSAN.

1. **Solubility.**—Test the solubility of *gum arabic* in the ordinary solvents (see page 27).

2. **Iodine Test.**—Add a drop of dilute iodine solution to a little *gum arabic* on a test-tablet. It resembles cellulose in giving *no color* with iodine.

3. **Hydrolysis of Gum Arabic.**—Introduce a little gum arabic into a test-tube, add 5–10 c.c. of strong hydrochloric acid (conc. HCl and water 1:1) and heat to boiling for 5–10 minutes. Cool, neutralize with potassium hydroxide and test by the Fehling or some other reduction test. A positive reaction should be obtained indicating that the gum arabic has been hydrolyzed by the acid with the production of a reducing substance. What is this reducing substance? How would you identify it?

#### EXPERIMENTS ON A GALACTAN.

1. **Solubility.**—Test the solubility of *agar-agar* in the ordinary solvents (see page 27). Observe its marked property of imbibing water (see page 255).

2. **Iodine Test.**—Add a drop of dilute iodine solution to a little *agar-agar* on a test-tablet. It resembles cellulose in giving *no color* with iodine.

3. **Hydrolysis of Agar-agar.**—Introduce a few pieces of *agar-agar* into a test-tube, add 5–10 c.c. of strong hydrochloric acid (conc. HCl and water 1:1) and heat to boiling for 5–10 minutes. Cool, neutralize with potassium hydroxide and test by the Fehling or some other reduction test. A positive reaction should be obtained indicating that

<sup>1</sup> Mendel: *Zentralblatt f. d. gesammte Phys. u. Path. des Stoffw.*, No. 17, 1, 1908.

<sup>2</sup> Schmidt: *Munch. med. Woch.*, 52, 1970, 1905.

<sup>3</sup> Morse: *Journal American Medical Ass'n.*, 55, 934, 1910.

<sup>4</sup> Einhorn: *Berl. klin. Woch.*, 49, 113, 1912.

the *agar-agar* has been hydrolyzed by the acid with the production of a reducing substance. What is this reducing substance? How would you identify it?

### REVIEW OF CARBOHYDRATES.

In order to facilitate the student's review of the carbohydrates, the preparation of a chart similar to the appended model is recommended.

#### MODEL CHART FOR REVIEW PURPOSES.

Carbo- hydrate.	Solubility.	Iodine Test.	Moore's Test.	Trommer's Test.	Fehling's Test.	Boettger's Test.	Nylander's Test.	Barfoed's Test.	Seliwanoff's Reaction.	Molisch's Reaction.	Mucic Acid Test.	Borhardt's Reaction.	Precipitation by Alcohol.	Osazone.	Rotation.	Diffusibility.	Fermentation.	Remarks.
Dextrose.																		
Lævulose.																		
Pentose																		
Maltose.																		
Iso-mal- tose.																		
Lactose.																		
Sucrose.																		
Starch.																		
Inulin.																		
Glycogen.																		
Dextrin.																		
Cellulose.																		
Gum Arabic																		
Agar-Agar																		

The signs + and - may be conveniently used to indicate positive and negative reaction. Only those carbohydrates which are of greatest importance from the standpoint of physiological chemistry have been included in the chart.

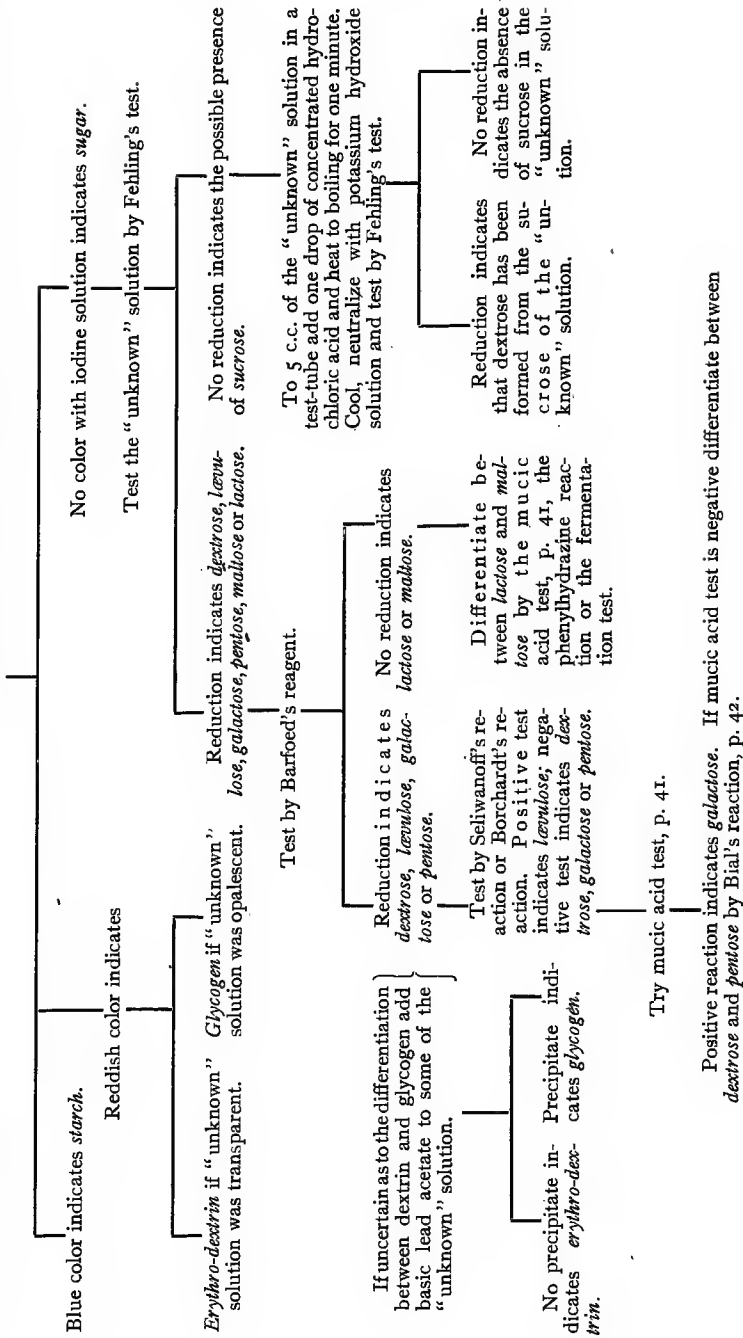
#### "UNKNOWN" SOLUTIONS OF CARBOHYDRATES.

At this point the student will be given several "unknown" solutions, each solution containing one or more of the carbohydrates studied. He will be required to detect, by means of the tests on the preceding pages, each carbohydrate constituent of the several "unknown" solutions and hand in, to the instructor, a written report of his findings, on slips furnished by the laboratory.

The scheme given on page 58 may be of use in this connection.

## SCHEME FOR THE DETECTION OF CARBOHYDRATES.

If the solution is alkaline, neutralize or make faintly acid with HCl. Test a few drops on a test-tablet, or in a test-tube with iodine solution.





## CHAPTER III.

### SALIVARY DIGESTION.

THE saliva is secreted by three pairs of glands, the submaxillary, sublingual, and parotid, reinforced by numerous small glands called buccal glands. The saliva secreted by each pair of glands possesses certain definite characteristics peculiar to itself. For instance, in man the parotid glands ordinarily secrete a thin, watery fluid, the submaxillary glands secrete a somewhat thicker fluid containing mucin, while the product of the sublingual glands has a more mucilaginous character. The saliva as collected from the mouth is the combined product of all the glands mentioned. The fact that there are pronounced variations in the composition of different fractions of saliva secreted by the same normal individual on a uniform diet has recently been emphasized by Lothrop and Gies.<sup>1</sup>

The saliva may be induced to flow by many forms of stimuli, such as *chemical, mechanical, electrical, thermal, and psychical*, the nature and amount of the secretion depending, to a limited degree, upon the particular class of stimuli employed as well as upon the character of the individual stimulus. For example, in experiments upon dogs it has been found that the mechanical stimulus afforded by dropping several pebbles into the animal's mouth caused the flow of but one or two drops of saliva, whereas the mechanical stimulus afforded by sand thrown into the mouth induced a copious flow of a thin watery fluid. Again, when ice-water or snow was placed in the animal's mouth no saliva was seen, while an acid or anything possessing a bitter taste, which the dog wished to reject, caused a free flow of the thin saliva. On the other hand, when articles of food were placed in the dog's mouth the animal secreted a thicker saliva having a higher mucin content—a fluid which would lubricate the food and assist in the passage of the bolus through the oesophagus. It was further found that by simply drawing the attention of the animal to any of the substances named above, results were obtained similar to those secured when the substances were actually placed in the animal's mouth. For example, when a pretense was made of throwing sand into the dog's mouth, a watery saliva was secreted, whereas food under the same conditions excited a thicker and more slimy secretion. The exhibition of dry food, in which the dog had no particular interest (dry bread) caused the

<sup>1</sup> Lothrop and Gies: *Journal of the Allied (Dental) Societies*, 6, 65, 1911.

secretion of a large amount of watery saliva, while the presentation of moist food, which was eagerly desired by the animal, called forth a much smaller secretion, slimy in character. These experiments show it to be rather difficult to differentiate between the influence of physiological and psychical stimuli.

The amount of saliva secreted by an adult in 24 hours has been variously placed, as the result of experiment and observation, between 1000 and 1500 c.c., the exact amount depending, among other conditions, upon the character of the food.

The saliva of adults ordinarily has a weak, alkaline reaction to litmus, but becomes acid, in some instances, 2-3 hours after a meal or during fasting. The saliva of the newborn is generally neutral to litmus, whereas that of infants, especially those breast-fed, is generally acid.<sup>1</sup> The alkalinity of saliva is due principally to di-sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) and its average alkalinity may be said to be equivalent to 0.08-0.1 per cent sodium carbonate. The saliva is the most dilute of all the digestive secretions, having an average specific gravity of 1.005 and containing only 0.5 per cent of solid matter. Among the solids are found albumin, globulin, mucin, urea, the enzymes salivary amylase (ptyalin), maltase, and peptide splitting enzymes; phosphates, and other inorganic constituents. Potassium thiocyanate, KSCN, is also generally present in the saliva. It has been claimed that this substance is present in greatest amount in the saliva of habitual smokers. The significance of thiocyanate in the saliva is not known; it probably comes from the ingested thiocyanates and from the breaking down of protein material. The attempts to show some relationship between tooth decay and the thiocyanate content of the saliva secreted into the mouth cavity have met with failure. The most recent experiments<sup>2</sup> indicate a virtual absence of such relationship.

The so-called tartar formation on the teeth is composed almost entirely of calcium phosphate with some calcium carbonate, mucin, epithelial cells, and organic debris derived from the food. The calcium salts are held in solution as acid salts, and are probably precipitated by the ammonia of the breath. The various organic substances just mentioned are carried down in the precipitation of the calcium salts.

The suggestion has been made that mucin is the salivary constituent "which is particularly influential in the development of local conditions favoring the onset of dental decay."<sup>3</sup>

The principal enzyme of the saliva is known as *salivary amylase* or *ptyalin*. This is an *amylolytic* enzyme (see page 4), so called because it possesses the property of transforming complex carbohydrates such as

<sup>1</sup> Allaria: *Monatsschr. für Kinderheilkunde*, 10, 179, 1911.

<sup>2</sup> Lothrop and Gies: *Journal of the Allied (Dental) Societies*, 6, 65, 1911.

<sup>3</sup> Id.; *Ibid.*, 5, No. 4, 1910.

starch and dextrin into simpler bodies. The action of salivary amylase is one of hydrolysis and through this action a series of simpler bodies are formed from the complex starch. The first product of the action of the ptyalin of the saliva upon starch paste is *soluble starch* (amidulin) and its formation is indicated by the disappearance of the opalescence of the starch solution. This body resembles true starch in giving a blue color with iodine. Next follows the formation, in succession, of a series of dextrans, called *erythro-dextrin*,  $\alpha$ -*achroo-dextrin*,  $\beta$ -*achroo-dextrin*, and  $\gamma$ -*achroo-dextrin*, the *erythro-dextrin* being formed directly from *soluble starch* and later being itself transformed into  $\alpha$ -*achroo-dextrin* from which in turn are produced  $\beta$ -*achroo-dextrin*,  $\gamma$ -*achroo-dextrin* and perhaps other dextrans. Accompanying each dextrin a small amount of iso-maltose is formed, the quantity of iso-maltose growing gradually larger as the process of transformation progresses. (Erythro-dextrin gives a red color with iodine, the other dextrans give no color.) The next stage is the transformation of the final dextrin into *iso-maltose* and subsequently the transformation of the iso-maltose into maltose, the latter being the principal end-product of the salivary digestion of starch. At this point a small amount of *dextrose* is formed from the maltose, through the action of the enzyme *maltase*.

Salivary amylase acts in alkaline, neutral, or combined acid solutions. It will act in the presence of relatively strong *combined* HCl (see page 126), whereas a trace (0.003 per cent to 0.006 per cent) of ordinary *free* hydrochloric acid will not only prevent the action but will destroy the enzyme. By sufficiently increasing the alkalinity of the saliva to litmus, the action of the salivary amylase is inhibited.

It has been claimed by Roger<sup>1</sup> that the activation of human saliva inactivated by the action of heat or hydrochloric acid could be brought about by the addition of traces of fresh human saliva. Very recent attempts<sup>2</sup> to verify this claim have met with failure.

It has recently been shown by Cannon that salivary digestion may proceed for a considerable period after the food reaches the stomach, owing to the slowness with which the contents are thoroughly mixed with the acid gastric juice and the consequent tardy destruction of the enzyme. Food in the pyloric end of the stomach is soon mixed with the gastric secretion, but food in the cardiac end is not mixed with the acid gastric juice for a considerable period of time, and in this region during that time salivary digestion may proceed undisturbed.

It has very recently been found that salivary amylase acts more efficiently when the saliva is *diluted*.<sup>3</sup> The optimum dilution for sodium

<sup>1</sup> Roger: *Rev. Gen. des. Sci.*, 18, 544, 1907.

<sup>2</sup> Bergeim and Hawk: Unpublished data.

<sup>3</sup> Bergeim and Hawk: Unpublished data.

chloride solution (0.3 per cent) was found to be about *four volumes*, whereas that for tap water<sup>1</sup> and distilled water was about *seven volumes*. These findings are of interest in connection with the more efficient utilization of ingested carbohydrate which has been found to accompany the drinking of large volumes of water at meal time.<sup>2</sup> Lipase also acts better in dilution.<sup>3</sup>

It has further been demonstrated very recently that the action of salivary amylase is inhibited in the presence of softened water.<sup>4</sup> The inhibitory factor was found to be *magnesium hydroxide*.<sup>5</sup> Electrolytes have an important influence upon the action of amylases. The Cl ion has a pronounced facilitating action (see Pancreatic Amylase).

The question of the *adaptation* of the salivary secretion to diet is one which has received considerable attention in recent years. It has been claimed, on the basis of experimental evidence,<sup>6</sup> that the continued feeding of a carbohydrate diet causes the secretion of a saliva which contains a higher concentration of salivary amylase and one which is therefore able to more efficiently digest the carbohydrate fed. On the other hand strong evidence<sup>7</sup> has been submitted that the amylase content of the saliva is not increased through the continued feeding of a carbohydrate diet. The balance of evidence is however opposed to adaptation. In general the concensus of opinion is *opposed to the adaptation of digestive secretions to diet*.

*Maltase*, sometimes called glucase, is the second enzyme of the saliva. The principal function of maltase is the splitting of maltose into dextrose. Besides occurring in the saliva it is also present in the pancreatic and intestinal juices. For experimental purposes the enzyme is ordinarily prepared from corn. The principles of the "reversibility" of enzyme action were first demonstrated in connection with maltase by Croft Hill.

The presence in the saliva of dipeptide- and tripeptide-splitting enzymes has recently been demonstrated.<sup>8</sup> Leucyl-glycyl-alanine was the tripeptide split whereas the cleavage of several dipeptids was brought about. The action is similar to that of intestinal erepsin (see Chapter VIII).

Microscopical examination of the saliva reveals salivary corpuscles,

<sup>1</sup> University of Illinois Water Supply.

<sup>2</sup> Mattill and Hawk: *Jour. Am. Chem. Soc.*, 33, 2019, 1911.

<sup>3</sup> Bradley: *Jour. Biol. Chem.*, 8, 251, 1910.

<sup>4</sup> Prepared by treating tap water with one-sixth its volume of saturated lime water, allowing to stand 24 hours and filtering.

<sup>5</sup> Bergeim and Hawk: Unpublished data.

<sup>6</sup> Neilson and Terry: *American Journal of Physiology*, 15, 406, 1905; Neilson and Lewis: *Journal of Biological Chemistry*, 4, 501, 1908.

<sup>7</sup> Mendel: *American Journal of the Medical Sciences*, Oct., 1909; Mendel and Underhill: *Journal of Biological Chemistry*, 3, 135, 1907. Mendel, Chapman and Blood: *Medical Record*, Aug. 27, 1910.

<sup>8</sup> Koelker: *Zeitschrift für physiol. Chem.*, 76, 27, 1911.

bacteria, food débris, epithelial cells, mucus, and fungi. In certain pathological conditions of the mouth, pus cells, and blood corpuscles may be found in the saliva.

### EXPERIMENTS ON SALIVA.

A satisfactory method of obtaining the saliva necessary for the experiments which follow is to chew a small piece of pure paraffin wax, thus stimulating the flow of the secretion, which may be collected in a small beaker. Filtered saliva is to be used in every experiment except for the microscopical examination.

1. **Microscopical Examination.**—Examine a drop of unfiltered saliva microscopically and compare with Fig. 19 below.

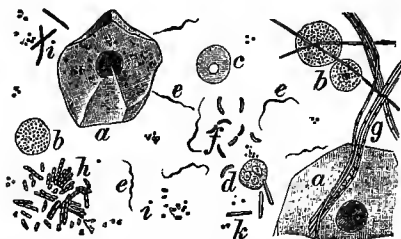


FIG. 19.—MICROSCOPICAL CONSTITUENTS OF SALIVA.

*a*, Epithelial cells; *b*, salivary corpuscles; *c* fat drops; *d*, leucocytes; *e*, *f* and *g*, bacteria; *h*, *i* and *k*, fission-fungi.

2. **Reaction.**—Test the reaction to litmus, phenolphthalein and Congo red.

3. **Specific Gravity.**—Partially fill a urinometer cylinder with saliva, introduce the urinometer, and observe the reading.

4. **Test for Mucin.**—To a small amount of saliva in a test-tube add 1–2 drops of dilute acetic acid. Mucin is precipitated.

5. **Biuret Test.**<sup>1</sup>—Render a little saliva alkaline with an equal volume of KOH and add a few drops of a *very dilute* (2–5 drops in a test-tube of water) copper sulphate solution. The formation of a purplish-violet color is due to mucin.

6. **Millon's Reaction.**<sup>2</sup>—Add a few drops of Millon's reagent to a little saliva. A light yellow precipitate formed by the mucin gradually turns red upon being *gently* heated.

7. **Preparation of Mucin.**—Pour 25 c.c. of saliva into 100 c.c. of 95 per cent alcohol, stirring constantly. Cover the vessel and allow the precipitate to stand at least 12 hours. Pour off the supernatant liquid, collect the precipitate on a filter and wash it, in turn, with alcohol and

<sup>1</sup> The significance of this reaction is pointed out on page 98.

<sup>2</sup> The significance of this reaction is pointed out on page 97.

ether. Finally dry the precipitate, remove it from the paper and make the following tests on the mucin: (a) Test its solubility in the ordinary solvents (see page 27); (b) Millon's reaction; (c) dissolve a small amount in KOH, and try the biuret test on the solution; (d) boil the remainder, with 10-25 c.c. of water to which 5 c.c. of dilute HCl has been added, until the solution becomes brownish. Cool, render alkaline with *solid* KOH, and test by Fehling's solution. A reduction should take place. Mucin is what is known as a conjugated protein or glycoprotein (see p. 94) and upon boiling with the acid the carbohydrate group in the molecule has been split off from the protein portion and its presence is indicated by the reduction of Fehling's solution.

8. **Inorganic Matter.**—Test for chlorides, phosphates, sulphates, and calcium. For chlorides, acidify with  $\text{HNO}_3$  and add  $\text{AgNO}_3$ . For phosphates, acidify with  $\text{HNO}_3$ , heat and add molybdic solution.<sup>1</sup> For sulphates, acidify with HCl and add  $\text{BaCl}_2$  and warm. For calcium, acidify with acetic acid,  $\text{CH}_3\text{COOH}$ , and add ammonium oxalate,  $(\text{NH}_4)_2\text{C}_2\text{O}_4$ .

9. **Viscosity Test.**—Place filter papers in two funnels, and to each add an equal quantity of starch paste (5 c.c.). Add a few drops of saliva to one lot of paste and an equivalent amount of water to the other. Note the progress of filtration in each case. Why does one solution filter more rapidly than the other?

10. **Test for Nitrites.**—Add 1-2 drops of dilute  $\text{H}_2\text{SO}_4$  to a little saliva and thoroughly stir. Now add a few drops of a potassium iodide solution and some starch paste. Nitrous acid is formed which liberates iodine, causing the formation of the blue iodide of starch.

11. **Thiocyanate Tests.**—(a) *Ferric Chloride Test.*—To a little saliva in a small porcelain crucible, or dish, add a few drops of dilute ferric chloride and acidify slightly with HCl. Red ferric thiocyanate forms. To show that the red coloration is not due to iron phosphate add a drop of  $\text{HgCl}_2$  when *colorless* mercuric thiocyanate forms.

(b) *Solera's Reaction.*—This test depends upon the liberation of iodine through the action of thiocyanate upon iodic acid. Moisten a strip of *starch paste-iodic acid* test paper<sup>2</sup> with a little saliva. If thiocyanate be present the test paper will assume a blue color, due to the liberation of iodine and the subsequent formation of the so-called iodide of starch.

<sup>1</sup> Molybdic solution is prepared as follows, the parts being by weight:

1 part molybdic acid.

4 parts ammonium hydroxide (sp. gr. 0.96).

15 parts nitric acid (sp. gr. 1.2).

<sup>2</sup> This test paper is prepared as follows: Saturate a good quality of filter paper with 0.5 per cent starch paste to which has been added sufficient iodic acid to make a 1 per cent solution of iodic acid and allow the paper to dry in the air. Cut it in strips of suitable size and preserve for use.

12. **Digestion of Starch Paste.**—To 25 c.c. of starch paste in a small beaker, add 5 drops of saliva and stir thoroughly. At intervals of a minute remove a drop of the solution to one of the depressions in a test-tablet and test by the iodine test. If the blue color with iodine still forms after 5 minutes, add another 5 drops of saliva. The opalescence of the starch solution should soon disappear, indicating the formation of *soluble starch* which gives a blue color with iodine. This body should soon be transformed into *erythro-dextrin* which gives a red color with iodine, and this in turn should pass into *achroo-dextrin* which gives no color with iodine. This is called the *achromic point*. When this point is reached test by Fehling's test to show the production of a reducing body. A positive Fehling's test may be obtained while the solution still reacts red with iodine inasmuch as some *iso-maltose* is formed from the soluble starch coincidentally with the formation of the *erythro-dextrin*. How long did it take for a complete transformation of the starch?

13. **Digestion of Dry Starch.**—In a test-tube shake up a small amount of *dry starch* with a little water. Add a few drops of saliva, mix well, and allow to stand. After 10–20 minutes filter and test the filtrate by Fehling's test. What is the result and why?

14. **Digestion of Inulin.**—To 5 c.c. of inulin solution in a test-tube add 10 drops of saliva and place the tube in the incubator or water-bath at 40° C. After one-half hour test the solution by Fehling's test.<sup>1</sup> Is any reducing substance present? What do you conclude regarding the salivary digestion of inulin?

15. **Influence of Temperature.**—In each of four tubes place about 5 c.c. of starch paste. Immerse one tube in cold water from the faucet, keep a second at room temperature, and place a third in the incubator or the water-bath at 40° C. Now add to the contents of each of these three tubes two drops of saliva and shake well; to the contents of the fourth tube add two drops of *boiled* saliva. Test frequently by the iodine test, using the test-tablet, and note in which tube the most rapid digestion occurs. Explain the results.

16. **Influence of Dilution.**<sup>2</sup>—Take a series of six test-tubes each containing 9 c.c. of water. Add 1 c.c. of saliva to tube 1 and shake thoroughly. Remove 1 c.c. of the solution from tube 1 to tube 2 and after mixing thoroughly remove 1 c.c. from tube 2 to tube 3. Continue in this manner until you have 6 saliva solutions of gradually decreasing strength. Now add starch paste in equal amounts to each tube, mix

<sup>1</sup> If the inulin solution gives a reduction before being acted upon by the saliva it will be necessary to determine the extent of the original reduction by means of a "check" test (see page 52).

<sup>2</sup> The technic of Wohlge-muth's method (see page 18) may be employed in this test if so desired.

very thoroughly, and place in the incubator or water-bath at  $40^{\circ}$  C. After 10–20 minutes test by both the iodine and Fehling's tests. In how great dilution does your saliva act?

**17. Influence of Acids and Alkalis.**—(a) *Influence of Free Acid.*—Prepare a series of six tubes in each of which is placed 4 c.c. of one of the following strengths of free HCl: 0.2 per cent, 0.1 per cent, 0.05 per cent, 0.025 per cent, 0.0125 per cent and 0.006 per cent. Now add 2 c.c. of starch paste to each tube and shake them thoroughly. Complete the solutions by adding 2 c.c. of saliva to each and repeat the shaking. The *total acidity* of this series would be as follows: 0.1 per cent, 0.05 per cent, 0.025 per cent, 0.0125 per cent, 0.006 per cent and 0.003 per cent. Place these tubes on the water-bath at  $40^{\circ}$  C. for 10–20 minutes. Divide the contents of each tube into two parts, testing one part by the iodine test and testing the other, after neutralization, by Fehling's test. What do you find?

(b) *Influence of Combined Acid (Protein Salt).*—Repeat the first three experiments of the above series using *combined* hydrochloric acid (see page 126) instead of the *free* acid. How does the action of the *combined acid* differ from that of the *free acid*? (For a discussion of combined acid see page 126.)

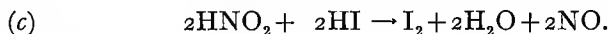
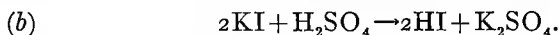
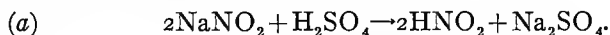
(c) *Influence of Alkali.*—Repeat the first four experiments under (a) replacing the HCl by 2 per cent, 1 per cent, 0.5 per cent and 0.25 per cent  $\text{Na}_2\text{CO}_3$ . Neutralize the alkalinity before trying the iodine test (see Starch, 5, page 50).

(d) *Nature of the Action of Acid and Alkali.*—Place 2 c.c. of saliva and 2 c.c. of 0.2 per cent HCl in a test-tube and leave for 15 minutes. Neutralize the solution, add 4 c.c. of starch paste and place the tube in the incubator or water-bath at  $40^{\circ}$  C. In 10 minutes test by the iodine and Fehling's tests and explain the result. Repeat the experiment, replacing the 0.2 per cent HCl by 2 per cent  $\text{Na}_2\text{CO}_3$ . What do you deduce from these two experiments?

**18. Influence of Metallic Salts, etc.**—In each of a series of tubes place 4 c.c. of starch paste and  $1/2$  c.c. of one of the solutions named below. Shake well, add  $1/2$  c.c. of saliva to each tube, thoroughly mix, and place in the incubator or water-bath at  $40^{\circ}$  C. for 10–20 minutes. Show the progress of digestion by means of the iodine and Fehling tests. Use the following chemicals: *Metallic salts*, 10 per cent lead acetate, 2 per cent copper sulphate, 5 per cent ferric chloride, 8 per cent mercuric chloride; *Neutral salts*, 10 per cent sodium chloride, 10 per cent magnesium sulphate, 3 per cent barium chloride, 10 per cent Rochelle salt. Also try the influence of 2 per cent carbolic acid, 95 per cent alcohol, and ether and chloroform. What are your conclusions?



19. **Excretion of Potassium Iodide.**—Ingest a small dose of potassium iodide (0.2 gram) contained in a gelatin capsule, quickly rinse out the mouth with water, and then test the saliva at once for iodine. This test should be negative. Make additional tests for iodine at 2-minute intervals. The test for iodine is made as follows: Take 1 c.c. of  $\text{NaNO}_2$  and 1 c.c. of dilute  $\text{H}_2\text{SO}_4$ <sup>1</sup> in a test-tube, add a little saliva directly from the mouth, and a small amount of starch paste. The formation of a blue color signifies that the potassium iodide is being excreted through the salivary glands. Note the length of time elapsing between the ingestion of the potassium iodide and the appearance of the first traces of the substance in the saliva. If convenient, the urine may also be tested. The chemical reactions taking place in this experiment are indicated in the following equations:



20. **Qualitative Analysis of the Products of Salivary Digestion.**—To 25 c.c. of the products of salivary digestion (saved from Experiment 12 or furnished by the instructor), add 100 c.c. of 95 per cent alcohol. Allow to stand until the white precipitate has settled. Filter, evaporate the filtrate to dryness, dissolve the residue in 5–10 c.c. of water and try Fehling's test (p. 32) and the phenylhydrazine reaction (see Dextrose, 3, page 28). On the dextrin precipitate try the iodine test (page 50). Also hydrolyze the dextrin as given under Dextrin, 4, page 53.

<sup>1</sup> Instead of this mixture a few drops of  $\text{HNO}_3$  possessing a yellowish or brownish color due to the presence of  $\text{HNO}_2$  may be employed.

## CHAPTER IV.

### PROTEINS :<sup>1</sup> THEIR DECOMPOSITION AND SYNTHESIS.

THE proteins are a class of substances, which in the light of our present knowledge, consist, *in the main*, of combinations of  $\alpha$ -amino-acids or their derivatives. These protein substances form the chief constituents of many of the fluids of the body, constitute the organic basis of animal tissue, and at the same time occupy a decidedly preëminent position among our organic food-stuffs. They are absolutely necessary to the uses of the animal organism for the continuance of life and they cannot be satisfactorily replaced in the diet of such an organism by any other dietary constituent either organic or inorganic. Such an organism may *exist* without protein food for a period of time, the length of the period varying according to the specific organism and the nature of the substitution offered for the protein portion of the diet. Such a period is, however, distinctly one of *existence* rather than one of normal life and one which is consequently not accompanied by such a full and free exercise of the various functions of the organism as would be possible upon an evenly balanced ration, *i. e.*, one containing the requisite amount of protein food. These protein substances are, furthermore, essential constituents of *all living cells* and therefore without them *vegetable life* as well as animal life is impossible.

The proteins, which constitute such an important group of substances, differ from the carbohydrates and fats very decidedly in elementary composition. In addition to containing *carbon, hydrogen, and oxygen*, which are present in fats and carbohydrates, the proteins invariably contain *nitrogen* in their molecule and generally *sulphur* also. Proteins have also been described which contain *phosphorus, iron, copper, iodine, manganese, and zinc*. The percentage composition of the more important members of the group of protein substances would fall within the following limits: C = 50-55 per cent, H = 6-7.3 per cent, O = 19-24 per cent, N = 15-19 per cent, S = 0.3-2.5 per cent, P = 0.4-0.8 per cent *when present*. When *iron, copper, iodine, manganese, or zinc* are present in the protein molecule they are practically without exception present only in *traces* and with the exception of *iodine* are probably not constituents of the protein molecule.<sup>2</sup>

<sup>1</sup> The term *proteid* has been very widely used by English-speaking scientists to signify the class of substances we have called *proteins*.

<sup>2</sup> Some investigators regard these elements as contaminations, or constituents of some non-protein substance combined with the protein.

Of all the various elements of the protein molecule, *nitrogen* is by far the most important. The human body needs nitrogen for the continuation of life, but it cannot use the nitrogen of the air or that in various other combinations as we find it in nitrates, nitrites, etc. However, in the protein molecule the nitrogen is present in a form which is utilizable by the body. The nitrogen in the protein molecule occurs in at least *four* different forms as follows:

- I. Monamino acid nitrogen.
- II. Diamino acid nitrogen or *basic* nitrogen.
- III. Amide nitrogen.
- IV. A guanidine residue.

The actual structure of the protein molecule is still unknown, and we have as yet no means by which its molecular weight can be even approximately established. The many attempts which have been made to determine this have led to very different results, some of which are given in the following table:

Globin	= 15000 — 16086
Oxyhæmoglobin	= 14800 — 15000 — 16655 — 16730

Of these figures, those given for oxyhæmoglobin deserve the most consideration, for these are based on the atomic ratios of the sulphur and iron contained in this substance. The simplest formula that can be calculated from analyses of oxyhæmoglobin, namely,  $C_{658}H_{1181}N_{207}S_2FeO_{210}$ , serves to show the great complexity of this substance.

The decomposition<sup>1</sup> of protein substances may be brought about by oxidation or hydrolysis, but inasmuch as the hydrolytic procedure has been productive of the more satisfactory results, that type of decomposition procedure alone is used at present. This hydrolysis of the protein molecule may be accomplished by acids, alkalis, or superheated steam, and in digestion by the action of the proteolytic enzymes. The character of the decomposition products varies according to the method utilized in tearing the molecule apart. Bearing this in mind, we may say that the decomposition products of proteins include *proteoses*, *peptones*, *peptides*, *carbon dioxide*, *ammonia*, *hydrogen sulphide*, and *amino acids*. These amino acids constitute a long list of important substances which contain nuclei belonging either to the *aliphatic*, *carbocyclic*, or *heterocyclic* series. The list includes *glycocoll*, *alanine*, *serine*, *phenylalanine*, *tyrosine*, *cystine*, *tryptophane*, *histidine*, *valine*, *arginine*, *leucine*, *isoleucine*, *lysine*, *aspartic acid*, *glutamic acid*, *proline*, *oxyproline*, and *diaminotrihydroxydodecanoic acid*.

<sup>1</sup> The terms "degradation," "dissociation," and "cleavage," are often used in this connection.

Of these amino acids, tyrosine and phenylalanine contain carbocyclic nuclei: histidine, proline, and tryptophane contain heterocyclic nuclei: and the remaining members of the list, as given, contain aliphatic nuclei. The amino acids are preëminently the most important class of protein decomposition products. These amino acids are all  $\alpha$ -amino acids, and, with the exception of glycocoll, are all optically active. Furthermore, they are amphoteric substances and consequently are able to form salts with both bases and acids. These properties are inherent in the  $\text{NH}_2$  and  $\text{COOH}$  groups of the amino acids.

The decomposition products of protein may be grouped as *primary* and *secondary* decomposition products. By *primary* products are meant those which exist as radicals within the protein molecule and which are liberated, upon cleavage of this molecule, with their carbon chains intact and the position of their nitrogen unaltered. The *secondary* products are those which result from the disintegration of the primary cleavage products. No matter what method is used to decompose a given protein molecule, the primary products are largely the same under all conditions.<sup>1</sup>

In the process of hydrolysis the protein molecule is gradually broken down and less complicated aggregates than the original molecule are formed, which are known as *proteoses*, *peptones*, and *peptides*, and which still possess true protein characteristics. Further hydrolysis causes the ultimate transformation of these substances, of a protein nature, into the amino acids of known chemical structure. In this decomposition the protein molecule is not broken down in a regular manner into  $1/2$ ,  $1/4$ ,  $1/8$  portions and the amino acids formed *in a group* at the termination of the hydrolysis. On the contrary, certain amino acids are formed very early in the process, in fact while the main hydrolytic action has proceeded no further than the proteose stage. Gradually the complexity of the protein portion undergoing decomposition is simplified by the splitting off of the amino acids and finally it is so far decomposed through previous cleavages that it yields only amino acids at the succeeding cleavage. In short, the general plan of the hydrolysis of the protein molecule is similar to the hydrolysis of starch. In the case of starch there is formed a series of dextrans of gradually decreasing complexity and coincidentally with the formation of each dextrin a small amount of sugar is split off and finally nothing but sugar remains. In the case of protein hydrolysis there is a series of proteins of gradually decreasing complexity produced and coincidentally with the formation of each new protein substance amino acids are split off and finally the sole products remaining are amino acids.

<sup>1</sup> Alkaline hydrolysis yields *urea* and *ornithine* which result from *arginine*, the product of acid hydrolysis.

Inasmuch as diversity in the method of decomposing a given protein does not result in an equally diversified line of decomposition products, but, on the other hand, yields products which are quite comparable in character, it may be argued that there are probably well-defined lines of cleavage in the individual protein molecule and that no matter what the force brought to bear to tear such a molecule apart, the disintegration, when it comes, will yield in every case certain definite fragments. These fragments may be called the "building stones" of the protein molecule, a term used by some of the German investigators. Take, for example, the decomposition of protein which may be brought about through the action of the enzyme *trypsin* of the pancreatic juice. When this enzyme is allowed to act upon a given protein, the latter is disintegrated in a series of definite cleavages, resulting in the formation of *proteoses*, *peptones*, and *peptides* in regular order, the peptides being the last of the decomposition products which possess protein characteristics. They are all built up from amino acids and are therefore closely related to these acids on the one side and to peptones on the other. We have *di-*, *tri-*, *tetra-*, *penta-*, *deca-*, and *poly-peptides* which are named according to the number of amino acids included in the peptide molecule. Following the peptides there are a diverse assortment of *monamino* and *diamino* acids which constitute the final products of the protein decomposition. These acids are devoid of any protein characteristics and are therefore decidedly different from the original substance from which they were derived. From a protein of huge molecular weight, a typical colloid, perhaps but slightly soluble, and entirely non-diffusible, we have passed by way of proteoses, peptones, and peptides to a class of simpler crystalline substances which are, for the most part, readily soluble and diffusible.

These amino acids after their production in the process of digestion, as just indicated, are synthesized within the organism to form protein material which goes to build up the tissues of the body. It is thus seen that the amino acids are of prime importance in the animal economy. It was formerly believed that these essential factors in metabolism and nutrition could not be produced within the animal organism from their elements, but were only yielded upon the hydrolysis of ingested protein of animal or vegetable origin. Recent experiments, however, by Abderholden and by Grafe and Schläpfer and others indicate that the nitrogen of food protein may in part be replaced by ammonium salts. Experiments by Osborne and others also indicate amino acid synthesis by animals.

There are formed, by life processes in both the animal and the vegetable kingdom certain transformation products of amino acids. Our knowledge regarding these has been advanced principally through the

efforts of Kutscher and his colleagues. This class of substances has been given the name *aporrhegmas*.<sup>1</sup> Among the aporrhegmas are included acids and bases formed in putrefaction as well as a number of similar compounds which have not been isolated from putrefaction mixtures but are formed normally in the plant or animal body. (For further discussion of aporrhegmas, see chapter on Putrefaction.)

Important data regarding the decomposition products of the protein molecule are given in the tables which follow.

COMPARISON OF THE DECOMPOSITION PRODUCTS OF PROTAMINES, AND OTHER PROTEINS.

Decomposition Product.	PROTAMINES. <sup>2</sup> (Per cent of total nitrogen of amino acid).						OTHER PROTEINS. (Per cent of amino acids in proteins.)						
	Secobrinc.	Cycloptericine.	Sturine.	$\alpha$ -cypyrinine.	$\beta$ -cypyrinine.	Clupeine.	Salmine.	Ghadin <sup>3</sup> (wheat).	Edestin <sup>4</sup>	Casein. <sup>5</sup>	Gelatin. <sup>5</sup>	Globin. <sup>7</sup>	*Zein. <sup>8</sup>
Glycocoll .....	.....	.....	.....	.....	.....	.....	.....	0.	3.8	0	16.5	0	0
Alanine .....	+	.....	+	.....	.....	+	.....	2.00	3.6	1.5	0.8	4.2	9.79
Valine.....	.....	.....	.....	+	+	+	1.65	3.34	6.2	7.2	1.0	.....	1.88
Leucine.....	.....	.....	+	.....	.....	.....	.....	6.62	14.5	9.4	2.1	29.0	19.55
Proline.....	3.8	.....	.....	.....	.....	+	4.3	13.22	4.1	6.7	5.2	2.3	9.04
Phenylalanine.....	.....	.....	.....	.....	.....	.....	.....	2.35	3.1	3.2	0.4	4.2	6.55
Aspartic acid.....	.....	.....	.....	.....	.....	.....	.....	0.58	4.5	1.4	0.56	4.4	1.71
Glutamic acid .....	.....	.....	.....	.....	.....	.....	.....	43.66	18.74	11.0	1.88	1.7	26.17
Serine.....	.....	.....	.....	.....	.....	+	3.25	0.13	0.33	0.5	0.4	0.6	1.02
Tyrosine.....	.....	2.2	.....	+	1.5	.....	.....	1.20	2.1	4.5	0	1.3	3.55
Arginine.....	88.8	67.7	63.5	8.7	28.0	88.0	89.2	3.16	14.2	4.84	7.62	5.4	1.55
Lysine.....	.....	.....	8.4	30.3	6.6	.....	.....	0	1.7	5.95	2.75	4.3	0
Histidine.....	.....	.....	11.8	.....	.....	.....	.....	0.61	2.2	2.50	0.40	11.0	0.82
Tryptophane.....	+	+	.....	.....	.....	.....	.....	1.0	+	1.5	0	+	0
Cystine.....	.....	.....	.....	.....	.....	.....	.....	0.45	1.00	0.065	0	0.3	?
Oxyproline .....	.....	.....	.....	.....	.....	.....	.....	?	2.0	0.23	6.4	1.0	?
Diaminotrihydroxydecanoic acid.	.....	.....	.....	.....	.....	.....	.....	.....	.....	0.75	.....	.....	?
Ammonia .....	.....	.....	.....	.....	.....	.....	.....	5.22	2.3	1.61	.....	.....	3.64

<sup>1</sup> Ackermann and Kutscher: *Zeit. physiol. Chem.*, 69, 263, 1910; Ackermann: *Ibid.*, 273; Engeland and Kutscher: *Ibid.*, 282.

<sup>2</sup> Kossel: *Zeit. physiol. Chem.*, 44, 347, 1905.

<sup>3</sup> Osborne and Guest: *Jour. Biol. Chem.*, 9, 425, 1911.

<sup>4</sup> Abderhalden, Kessel and others.

<sup>5</sup> Abderhalden, Fischer, Möner and others.

<sup>6</sup> Fischer, Levene and Aders: *Zeit. physiol. Chem.*, 35, 70, 1902; also Levene and Beatty: *Ibid.*, 49, 252, 1906.

<sup>7</sup> Abderhalden: *Zeit. physiol. Chem.*, 37, 484, 1903.

<sup>8</sup> Osborne and Liddle, *Am. Jour. Physiol.*, 26, 295, 1910.

\* This unique and important protein has probably been more carefully analyzed than any other.

AMINO ACIDS OBTAINED UPON DECOMPOSITION OF THE PROTEIN MOLECULE.

Nomenclature.	Nucleus. <sup>1</sup>	Formula.	Class.	Optical activity.	Discovered.		Maximum yield.			
					By	Date.	Per cent.	Obtained by	Date.	From decomposition of
<i>Arginine</i> : guanidin- $\alpha$ -amino-valerianic acid.	A	$C_9H_{14}N_4O_2$ .....	Monobasic, diamino.	<i>d</i>	Schulze and Steiger.	1886	89.20 <sup>2</sup>	Kossel and Pringl.	1906	Salmine.
<i>Alanine</i> : $\alpha$ -amino-propionic acid.	A	$C_3H_7NO_2$ .....	Monobasic, monamino.	<i>d</i>	Schützenberger and Bourgeois.	1875	24.00	Aberghalden and Spack.	1909	Tussah silk fibroin.
<i>Lysine</i> : $\alpha$ - $\epsilon$ -diaminocaproic acid.	A	$C_9H_{14}N_2O_2$ .....	Monobasic, diamino.	<i>d</i>	Drechsel.....	1889	28.80	Kossel and Dakin.	1904	$\alpha$ -Cyprinine.
<i>Histidine</i> : $\alpha$ -imidazolamino- $\beta$ -propionic acid.	C	$C_8H_9N_3O_2$ .....	Monobasic, monamino.	<i>l</i>	Kossel.....	1896	12.90	Kossel and Kutscher.	1900	Sturine.
<i>Proline</i> : $\alpha$ -pyrrolidincarboxylic acid.	H. P.	$C_5H_9NO_2$ .....	Pyrryl derivative.	<i>l</i>	Fischer.....	1901	13.73	Osborne and Clapp.	1907	Hordein.
<i>Tryptophane</i> : indol- $\alpha$ -amino-propionic acid.	H. I.	$C_{11}H_{12}N_2O_2$ .....	Indol derivative.	<i>l</i>	Hopkins and Cole.	1901	1.5(?)	Fischer.....	1901	Casein.
<i>Oxyproline</i> : oxy- $\alpha$ -pyrrolidincarboxylic acid.	H. P.	$C_5H_9NO_3$ .....	Pyrryl derivative.	<i>l</i>	Fischer.....	1902	6.4	Levene and Beatty.	1906	Gelatin.
<i>Isoleucine</i> : $\alpha$ -amino- $\beta$ -methyl- $\beta$ -ethyl-propionic acid.	A	$C_8H_{13}NO_2$ .....	Monobasic, monamino.	<i>d</i>	Ehrlich.....	1903	3.0	Levene, Van Slyke and Birchard.	1910	Fibrin-Heteroproteose.
<i>Diaminotrihydroxydodecanoic acid</i> .	A	$C_{12}H_{23}N_2O_5$ .....	Oxydiamino...	<i>l</i>	Fischer and Aberghalden, Skraup.	1904	0.75	Fischer and Aberghalden.	1904	Casein.

<sup>1</sup> A = Aliphatic; C = Carbocyclic; H. I. = Heterocyclic (Indole ring); H. P. = Heterocyclic (Pyrryl ring). <sup>2</sup> Per cent. of total nitrogen.

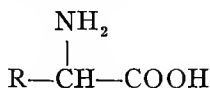
AMINO ACIDS OBTAINED UPON DECOMPOSITION OF THE PROTEIN MOLECULE.—Continued.

Nomenclature.	Nucleus. <sup>1</sup>	Formula.	Class.	Optical activity.	Discovered.		Maximum yield.			
					By	Date.	Per cent.	Obtained by	Date.	From decomposition of
<i>Cystine</i> : $\alpha$ -diamino- $\beta$ -dithiolyacetyl acid.	A	$C_9H_{12}O_4N_4S_2 \dots$	(Disulphide of) monobasic, monamino.	<i>l</i>	Wollaston.....	1810	13.92	Mörner.....	1901	Human hair.
<i>Leucine</i> : $\alpha$ -amino-isobutylic-acetic acid.	A	$C_6H_{13}NO_2 \dots$	Monobasic, monamino.	<i>l</i>	Proust.....	1818	29.04	Aberhalden.	1903	Globin.
<i>Glycocoll</i> : amino acetic acid.	A	$C_2H_3NO_2 \dots$	Monobasic, monamino.	<i>z</i>	Braconot.....	1820	37.5	Aberhalden and Behrend.	1909	Canton silk.
<i>Aspartic acid</i> : amino-succinic acid.	A	$C_4H_7NO_4 \dots$	Dibasic, monamino.	<i>l</i>	Plisson.....	1827	23.80	Aberhalden and Strauss.	1906	Spongin.
<i>Tyrosine</i> : p-oxy- $\beta$ -phenyl- $\alpha$ -amino-propionic acid.	C	$C_9H_{11}NO_3 \dots$	Monobasic, monamino.	<i>l</i>	Liebig.....	1846	10.06	Kutscher.....	1903	Zein.
<i>Valine</i> : $\alpha$ -amino isovalerianic acid.	A	$C_5H_{11}NO_2 \dots$	Monobasic, monamino.	<i>d</i>	Gorup-Besanez.	1856	7.20	Osborne and Guest.	1911	Casein.
<i>Serine</i> : $\alpha$ -amino- $\beta$ -hydroxy-propionic acid	A	$C_3H_7NO_3 \dots$	Oxymonamino.	<i>l</i>	Cramer.....	1865	7.80	Kossel and Dakin.	1904	Salmine.
<i>Glutamic acid</i> : $\alpha$ -amino-normal-glutaric acid.	A	$C_6H_9NO_4 \dots$	Dibasic, monamino.	<i>d</i>	Ritthausen.....	1866	43.66	Osborne and Guest.	1911	Gliadin.
<i>Phenylalanine</i> : $\beta$ -phenyl- $\alpha$ -amino-propionic acid.	C	$C_9H_{11}NO_2 \dots$	Monobasic, monamino	<i>l</i>	Schulze and Barbieri.	1877	6.96	Langstein.....	1903	Zein.

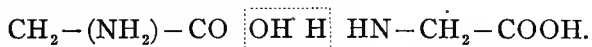
<sup>1</sup> A = Aliphatic; C = Carbocyclic; H. I. = Heterocyclic (Indole ring); H. P. = Heterocyclic (Pyrrol ring).



When we examine the formulas of the principal members of the crystalline end-products of protein decomposition we note that they are invariably acids, as has already been mentioned, and contain an  $\text{NH}_2$  group in the  $\alpha$  position. This relation of the  $\text{NH}_2$  group to the acid radical is *constant*, no matter what other groups or radicals are present. We may have straight *chains* as in alanine and glutamic acid, the *benzene ring* as in phenylalanine, or we may have *sulphurized bodies* as in cystine and still the formula is always of the same type, *i. e.*,



It is seen that this characteristic grouping in the amino acid provides each one of these ultimate fragments of the protein molecule with both a strong *acid* and a strong *basic* group. For this reason it is theoretically possible for a large number of these amino acids to combine and the resulting combinations may be very great in number, since there is such a varied assortment of the acids. The protein molecule, which is of such mammoth proportions, is probably constructed on a foundation of this sort. Of late much valuable data have been collected regarding the synthetic production of protein substances, the leaders in this line of investigation being Fischer and Abderhalden. After having gathered a mass of data regarding the final products of the protein decomposition and demonstrating that amino acids were the ultimate results of the various forms of decomposition, these investigators, and notably Fischer, set about in an effort to form, from these amino acids, by synthetic means, substances which should possess protein characteristics. The simplest of these bodies formed in this way was synthesized from two molecules of glycoll with the liberation of water, thus:



The body thus formed is a *dipeptide*, called *glycyl-glycine*. In an analogous manner may be produced *leucyl-leucine*, through the synthesis of two molecules of leucine or *leucyl-alanyl-glycine* through the union of one molecule of leucine, one of alanine, and one of glycoll. By this procedure Fischer and his pupils have been able to make a large number of peptides containing varied numbers of amino-acid radicals, the name *polypeptides* being given to the whole group of synthetic substances thus formed. The most complex polypeptide yet produced is one containing fifteen glycoll and three leucine residues.

Notwithstanding the fact that most synthetic polypeptides are produced through a union of amino acids by means of their imide bonds, it

must not be imagined that the protein molecule is constructed from amino acids linked together in straight chains in a manner analogous to the formation of simple peptides, such as glycyl-glycine. The molecular structure of the proteins is much too complex to be explained upon any such simple formation as that. There must be a variety of linkings, since there is a varied assortment of decomposition products of totally different structure.

Many of these synthetic bodies respond to the biuret test, are precipitated by phosphotungstic acid, and behave, in other ways, as to leave no doubt as to their protein characteristics. For instance, a number of amino acids each possessing a *sweet* taste have been synthesized in such a manner as to yield a polypeptide of *bitter* taste, a well-known characteristic of peptones. From the fact that the polypeptides formed in the manner indicated have free acidic and basic radicals we gather the explanation of the amphoteric character of true proteins.

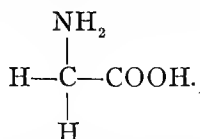
For the benefit of those especially interested in such matters a photograph of the Fischer apparatus (Fig. 23, page 80) used in the fractional distillation, *in vacuo*, of the esters of the decomposition products of the proteins, as well as micro-photographs and drawings of preparations of several of these decomposition products (Figs. 20 to 32, pages 77 to 89) are introduced. For the preparations and the photograph of the apparatus the author is indebted to Dr. T. B. Osborne, of New Haven, Conn., who has made many important observations upon the hydrolysis of proteins. The reproduction of the crystalline form of some of the more recent of the products may be of interest to those viewing the field of physiological chemistry from other than the student's aspect.

An extended discussion of the various decomposition products being out of place in a book of this character, we will simply make a few general statements in connection with the primary decomposition products.

## DISCUSSION OF THE PRODUCTS.

**Ammonia,  $\text{NH}_3$ .**—Ammonia is an important decomposition product of all proteins and probably arises from an amide group combined with a carboxyl group of some of the amino acids. It is possible that the dibasic acids, aspartic and glutamic, furnish most of these carboxyl groups. This is indicated by the more or less close relationship which exists between the amount of ammonia and that of the dibasic acids which the several proteins yield upon decomposition. The elimination of the ammonia from proteins under the action of acids and alkalis is very similar to that from amides like asparagine.

**Glycocoll**,  $C_2H_5NO_2$ .—Glycocoll, or *amino acetic acid*, is the simplest of the amino acids and has the following formula:



Glycocoll, as the formula shows, contains no asymmetric carbon atom, and is the only amino acid yielded by protein decomposition which is *optically inactive*. Glycocoll and leucine were among the first decomposition products of proteins to be discovered. Upon administering benzoic acid to animals the output of hippuric acid in the urine is greatly increased,

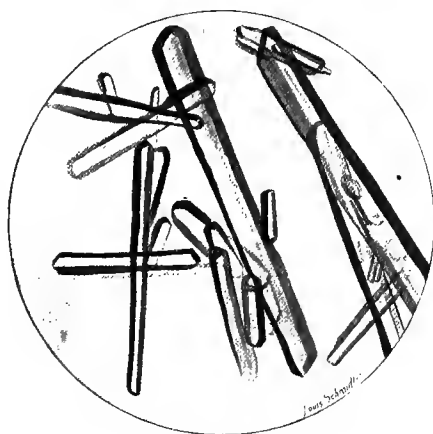
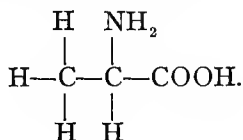


FIG. 20.—GLYCOCOLL ESTER HYDROCHLORIDE.

thus showing a synthesis of benzoic acid and glycocoll in the organism (see page 168, Chapter IX). Glycocoll, ingested in small amount, is excreted in the urine as urea, whereas if administered in excess it appears in part unchanged in the urine. It is usually separated from the mixture of protein decomposition products as the hydrochloride of the ester. The crystalline form of this compound is shown in Fig. 20.

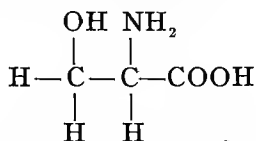
**Alanine**,  $C_3H_7NO_2$ .—Alanine is *α-amino-propionic acid*, and as such it may be represented structurally as follows:



Obtained from protein substances, alanine is dextro-rotatory, is very soluble in water, and possesses a sweet taste. Tyrosine, phenylalanine,

cystine, and serine are derivatives of alanine. This amino acid has been obtained from nearly all proteins examined. Its absence from those proteins from which it has not been obtained has not been proven. Most proteins yield relatively small amounts of alanine.

**Serine**,  $C_3H_7NO_3$ .—Serine is  $\alpha$ -amino- $\beta$ -hydroxy-propionic acid and possesses the following structural formula:



Serine obtained from proteins is lævo-rotatory, possesses a sweet taste, and is quite soluble in water. Serine is not obtained in quantity from most proteins, but is yielded abundantly by *silk glue*. Owing to the difficulty of separating serine it has not been found in a number of proteins in which it probably occurs. Serine crystals are shown in Fig. 21, below.

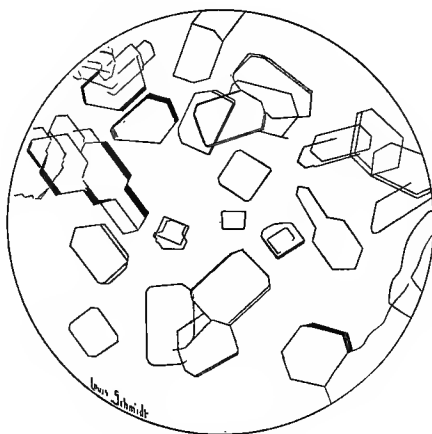
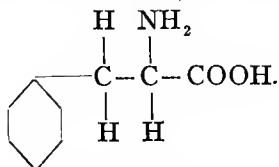


FIG. 21.—SERINE.

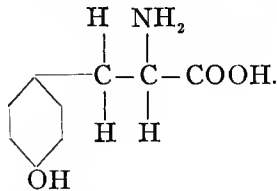
**Phenylalanine**,  $C_9H_{11}NO_2$ .—This product is  $\beta$ -phenyl- $\alpha$ -amino-propionic acid, and may be represented graphically as follows:



The lævo-rotatory form is obtained from proteins. Phenylalanine has been obtained from all the proteins examined except from the protamines and some of the albuminoids. The yield of this body from the decom-

position of proteins is frequently greater than the yield of tyrosine. The crystalline form of phenylalanine is shown in Fig. 22.

**Tyrosine**,  $C_9H_{11}NO_3$ .—Tyrosine, one of the first discovered end-products of protein decomposition, is the amino acid, *p*-oxy- $\beta$ -phenyl- $\alpha$ -amino-propionic acid. It has the following formula:



The tyrosine which results from protein decomposition is usually lævotatory. Tyrosine is one of the end-products of tryptic digestion and usually separates in conspicuous amount early in the process of digestion.

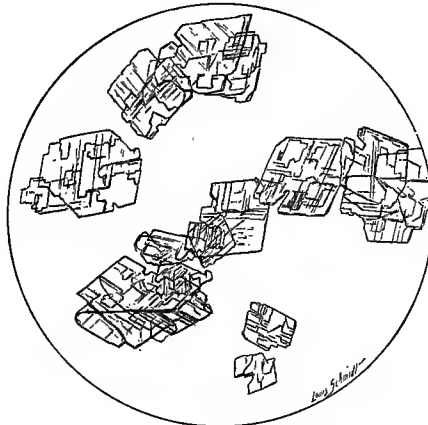


FIG. 22.—PHENYLALANINE.

It does not occur, however, as an end-product of the decomposition of gelatin.

Tyrosine is found in old cheese, and derives its name from this fact. It crystallizes in tufts, sheaves, or balls of fine needles, which decompose at 295° C. and are sparingly soluble in cold (1-2454) water, but much more so in boiling (1-154) water. Tyrosine forms soluble salts with alkalis, ammonia, or mineral acids, and is soluble, with difficulty, in acetic acid. It responds to Millon's reaction, thus showing the presence of the hydroxyphenyl group, but gives no other protein test. The aromatic groups present in tyrosine, phenylalanine, and tryptophane cause proteins to yield a positive xanthoproteic reaction. In severe cases of typhoid fever and smallpox, in acute yellow atrophy of the liver, and in

acute phosphorus poisoning, tyrosine has been found in the urine. Tyrosine crystals are shown in Fig. 24, page 81.

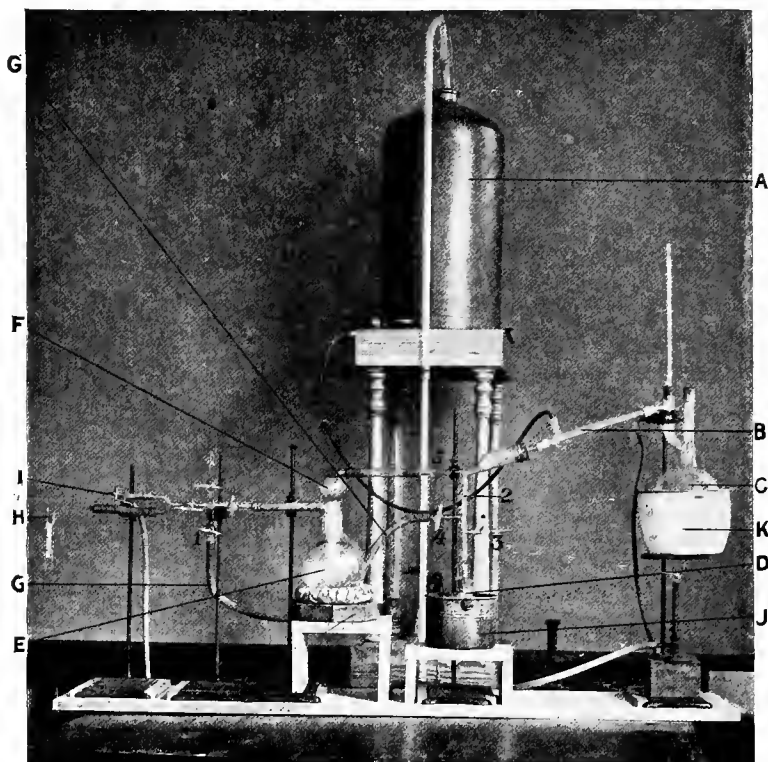
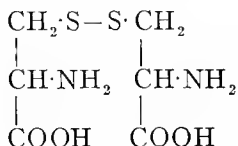


FIG. 23.—FISCHER APPARATUS.

Reproduced from a photograph made by Prof. E. T. Reichert, of the University of Pennsylvania. The negative was furnished by Dr. T. B. Osborne, of New Haven, Conn.

A, Tank into which freezing mixture is pumped and from which it flows through the condenser, B; C, flask from which the esters are distilled, the distillate being collected in D; E, a Dewar flask containing liquid air serving as a cooler for condensing tube F; G and G', tubes leading to the Geryck pump by which the vacuum is maintained; I, tube leading to a McLeod gauge (not shown in figure); J, a bath containing freezing mixture in which the receiver D is immersed; K, a bath of water during the first part of the distillation and of oil during the last part of the process; 1-5, stop cocks which permit the cutting out of different parts of the apparatus as the procedure demands.

**Cystine**,  $C_6H_{12}O_4N_2S_2$ .—Friedmann has recently shown cystine to be  $\alpha$ -diamino- $\beta$ -dithiolactyl acid and to possess the following structural formula:



Cystine is the principal sulphur-containing body obtained from the decomposition of protein substances. It is obtained in greatest amount as a decomposition product of such keratin-containing tissues as horn,

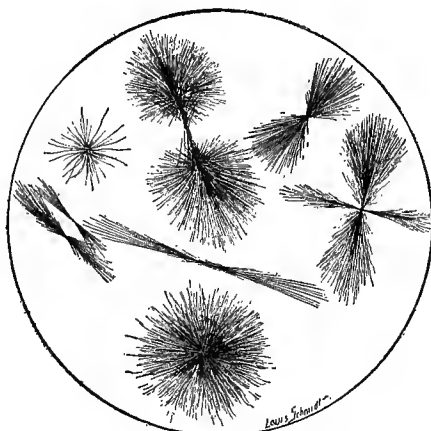


FIG. 24.—TYROSINE.

hoof, and hair. Cystine occurs in small amount in normal urine and is greatly increased in quantity under certain pathological conditions. It crystallizes in thin, colorless, hexagonal plates which are shown in Fig. 25. Cystine is very *slightly* soluble in water but its salts, with both bases and acids, are *readily* soluble in water. It is lævo-rotatory.

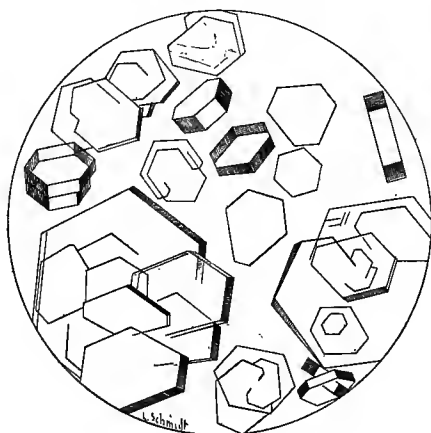
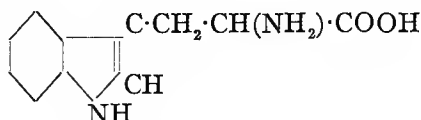


FIG. 25.—CYSTINE.

It was formerly claimed that cystine occurred in two forms, *i. e.*, stone-cystine and protein-cystine and that these two forms are distinct in their properties. This view is incorrect.

For a discussion of cystine sediments in urine see Chapter XX.

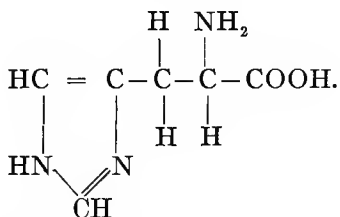
**Tryptophane**,  $C_{11}H_{12}N_2O_2$ .—Recently Ellinger and Flamand have shown that tryptophane possesses the following formula :



It is therefore *indol- $\alpha$ -amino-propionic acid*. Tryptophane is the *mother-substance of indole, skatole, skatole acetic acid and skatole carboxylic acid*, all of which are formed as *secondary* decomposition products of proteins. Its presence in protein substances may be shown by means of the Adamkiewicz reaction or the Hopkins-Cole reaction (see page 98). It may be detected in a tryptic digestion mixture through its property of giving a violet color-reaction with bromine water. Tryptophane is yielded by nearly all proteins, but has been shown to be *entirely absent from zein*, the prolamin (alcohol-soluble protein) of maize and also from gelatin.

Upon being heated to  $285^\circ \text{C}$ . tryptophane decomposes with the evolution of gas.

**Histidine**,  $C_6H_9N_3O_2$ .—Histidine is  *$\alpha$ -amino- $\beta$ -imidazol-propionic acid* with the following structural formula :



The histidine obtained from proteins is *lævo-rotatory*. It has been obtained from all the proteins thus far examined, the majority of them yielding about 2.5 per cent of the amino acid. However, about 11 per cent was obtained by Abderhalden from *globin*, the protein constituent of oxyhæmoglobin and about 13 per cent by Kossel and Kutscher from the protamine *sturine*.

Crystals of histidine dichloride are shown in Fig. 26, page 83.

*Knoop's Color Reaction for Histidine*.—To an aqueous solution of histidine or a histidine salt in a test-tube add a little bromine water. A yellow coloration develops in the cold and upon further addition of bromine water becomes permanent. If the tube be heated,<sup>1</sup> the color will disappear and will shortly be replaced by a faint red coloration which

<sup>1</sup> The same reaction will take place in the cold more slowly.



gradually passes into a deep wine red. Usually black, amorphous particles separate out and the solution becomes turbid.

The reaction cannot be obtained in solutions containing free alkali. It is best to use such an amount of bromine as will produce a permanent yellow color in the cold. The use of a less amount of bromine than this produces a weak coloration whereas an excess of bromine prevents the reaction. The test is not very delicate, but a characteristic reaction may always be obtained in 1:1000 solutions. The only histidine derivative which yields a similar coloration is imidazoylethylamine, and the reaction

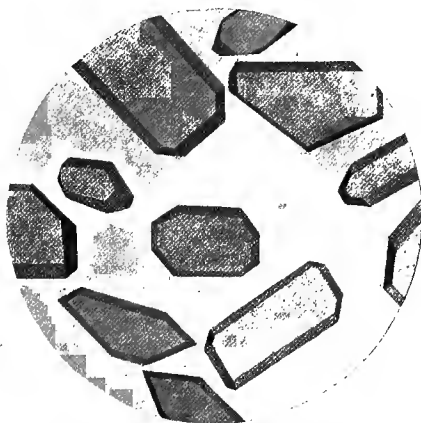
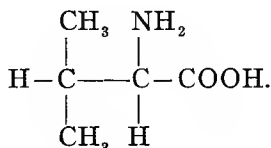


FIG. 26.—HISTIDINE DICHLORIDE.

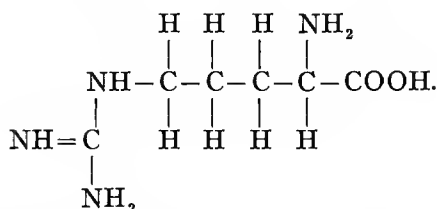
in this case is rather weak as compared with the color obtained with histidine or histidine salts.

**Valine**,  $C_5H_{11}NO_2$ .—The amino-valerianic acid obtained from proteins is *α-amino-isovalerianic acid*, and as such bears the following formula:



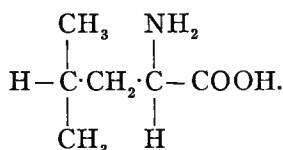
It closely resembles leucine in many of its properties, but is more soluble in water. It is a difficult matter to identify valine in the presence of leucine and isoleucine inasmuch as these amino acids crystallize together in such a way that the combination persists even after repeated recrystallizations. Valine is dextro-rotatory.

**Arginine**,  $C_6H_{14}N_4O_2$ .—Arginine is *guanidine-α-amino-valerianic acid* and possesses the following structural formula:



It has been obtained from every protein so far subjected to decomposition. The arginine obtained from proteins is dextro-rotatory, and has pronounced basic properties, reacts strongly alkaline to litmus, and forms stable carbonates. Because of these facts, Kossel considers arginine to be the nucleus of the protein molecule. It is obtained in widely different amounts from different proteins, over 85 per cent of certain protamines having been obtained in the form of this amino acid. It is claimed that in the ordinary metabolic activities of the animal body arginine gives rise to urea. While this claim is probably true, it should, at the same time, be borne in mind that the greater part of the protein nitrogen is eliminated as urea and that, therefore, but a very small part can arise from arginine.

**Leucine**,  $\text{C}_6\text{H}_{13}\text{NO}_2$ .—Leucine is an abundant end-product of the decomposition of protein material, and was one of the first of these products to be discovered. It is *α-amino-isobutyl-acetic acid*, and therefore has the following formula:



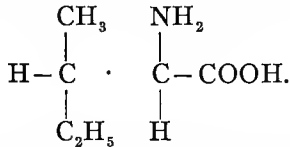
The leucine which results from protein decomposition is *l*-leucine. Leucine is present *normally* in the pancreas, thymus, thyroid, spleen, brain, liver, kidneys, and salivary glands. It has been found *pathologically* in the urine (in acute yellow atrophy of the liver, in acute phosphorus poisoning, and in severe cases of typhoid fever and smallpox), and in the liver, blood, and pus.

Pure leucine crystallizes in thin, white, hexagonal plates. Crystals of pure leucine are reproduced in Fig. 27. It is rather easily soluble in water (46 parts), alkalis, ammonia, and acids. On rapid heating to  $295^\circ\text{C}$ ., leucine decomposes with the formation of carbon dioxide, ammonia, and amylamine. Aqueous solutions of leucine obtained from proteins are *lævo*-rotatory, but its acid or alkaline solutions are *dextro*-rotatory. So-called impure leucine<sup>1</sup> is a slightly refractive substance, which generally crystal-

<sup>1</sup> These balls of so-called *impure* leucine do contain considerable leucine, but inasmuch as they may contain many other things it is a bad practice to allude to them as *leucine*.

lizes in balls having a radial structure, or in aggregations of spherical bodies, Fig. 109, Chapter XX.

**Isoleucine**,  $C_6H_{13}NO_2$ .—Isoleucine is  $\alpha$ -amino- $\beta$ -methyl- $\beta$ -ethyl-propionic acid, and possesses the following structural formula:



This amino acid was discovered by Ehrlich in 1903. Its presence has been established among the decomposition products of only a few proteins

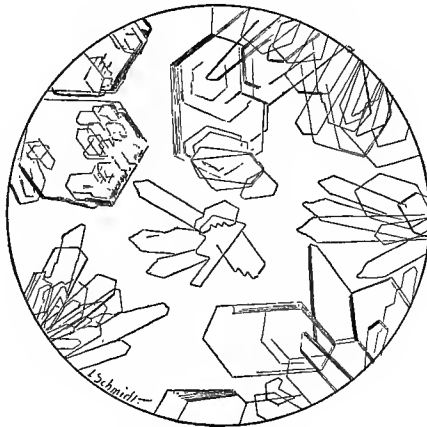
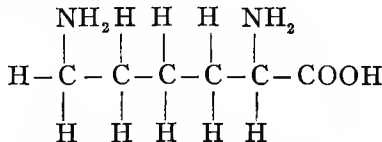


FIG. 27.—LEUCINE.

although it probably occurs among those of many or most of them. Ehrlich has shown that the *d*-amyl alcohol which is produced by yeast fermentation originates from isoleucine and the isoamylalcohol originates from leucine. Isoleucine is dextro-rotatory.

**Lysine**,  $C_6H_{14}N_2O_2$ .—The three bodies, lysine, arginine, and histidine, are frequently classed together as the *hexone bases*. Lysine was the first of the bases discovered. It is  $\alpha$ - $\epsilon$ -diamino-caproic acid and hence possesses the following structure:



It is dextro-rotatory and is found in relatively large amount in casein and gelatin. Lysine is obtained from nearly all proteins, but is absent from the vegetable proteins which are soluble in strong alcohol. It is the mother-substance of cadaverin and has never been obtained in crystalline

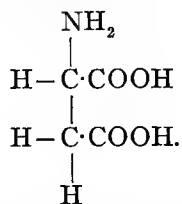
form. Lysine is usually obtained as the picrate which is sparingly soluble in water and crystallizes readily. These crystals are shown in Fig. 28.

FIG. 28.—LYSINE PICRATE.



FIG. 29.—ASPARTIC ACID.

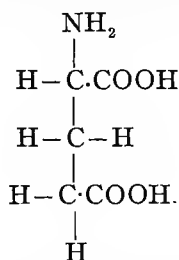
**Aspartic Acid,  $C_4H_7NO_4$ .**—Aspartic acid is *amino-succinic acid* and has the following structural formula:



The amide of aspartic acid, *asparagine*, is very widely distributed in the vegetable kingdom. The crystalline form of aspartic acid is exhibited in Fig. 29.

Aspartic acid has been found among the decomposition products of all the proteins examined, *except the protamines*. It has not been obtained, however, in very large proportion from any of them. The aspartic acid obtained from protein is lævo-rotatory.

**Glutamic Acid,  $C_5H_9NO_4$ .**—This acid is  *$\alpha$ -amino-normal-glutaric acid* and as such bears the following graphic formula:



Glutamic acid is yielded by all the proteins thus far examined, except the protamines, and by most of these in larger amount than any other of

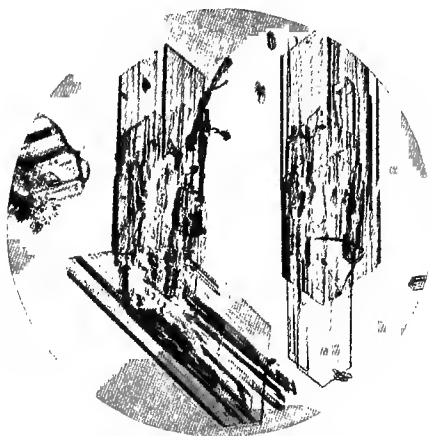


FIG. 30.—GLUTAMIC ACID.

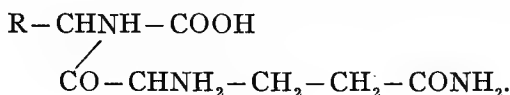
Reproduced from a micro-photograph made by Prof. E. T. Reichert, of the University of Pennsylvania.

their decomposition products. It is yielded in especially large proportion by most of the proteins of seeds, 43.66 per cent having been obtained very recently by Osborne and Guest<sup>1</sup> by the hydrolysis of gliadin, the prolamin of wheat. This is the largest amount of any single decomposition product yet obtained from any protein except the protamines.

Glutamic acid and aspartic acid are the only dibasic acids which have thus far been obtained as decomposition products of proteins. As there is an apparent relation between the proportion of these acids and that of

<sup>1</sup> Osborne and Guest; *Jour. Biol. Chem.*, 9, 425, 1911

ammonia which the different proteins yield it is possible that one of the carboxyl groups of these acids is united with  $\text{NH}_2$  as an amide, the other carboxyl group being united in polypeptide union (see page 75) with some other amino acid. This might be represented by the following formula:



It has not been definitely proven, however, that this form of linking actually occurs.

The glutamic acid, yielded by proteins upon hydrolysis, is dextro-rotatory. Crystals of glutamic acid are reproduced in Fig. 30, p. 87.

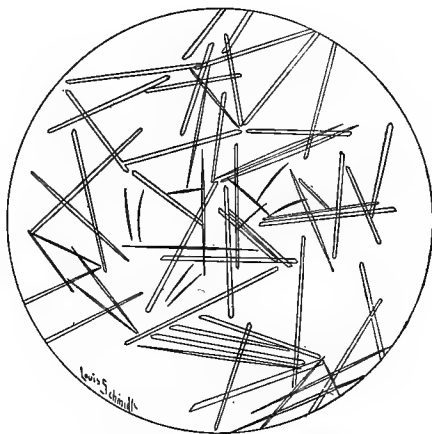
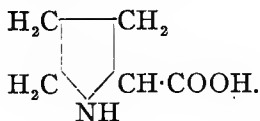


FIG. 31.—L- $\alpha$ -PROLINE.

**Proline**,  $\text{C}_5\text{H}_9\text{NO}_2$ .—Proline is  $\alpha$ -pyrrolidine-carboxylic acid and possesses the following graphic structure:



Proline was first obtained as a decomposition product of casein. Proline obtained from proteins is lævo-rotatory and is the only protein decomposition product which is readily soluble in alcohol. It is also one of the few heterocyclic compounds obtained from proteins. Proline has been found among the decomposition products of all proteins except the protamines. The maximum yield reported is 13.73 per cent obtained by Osborne and Clapp from the hydrolysis of hordein. More recently Fischer and

Boehner<sup>1</sup> reported having obtained 7.7 per cent from the hydrolysis of gelatin. The crystalline form of *lævo-α-proline* is shown in Fig. 31, and the copper salt of proline is represented by a micro-photograph in Fig. 32, below. The crystals of the copper salt have a deep blue color, but when they lose their water of crystallization they assume a characteristic violet color.

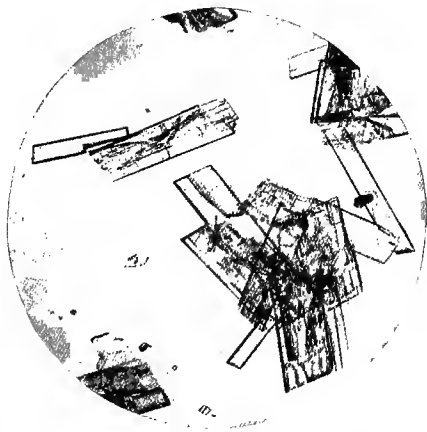


FIG. 32.—COPPER SALT OF PROLINE.

Reproduced from a micro-photograph made by Prof. E. T. Reichert, of the University of Pennsylvania.

**Oxyproline**,  $C_5H_9NO_3$ .—Oxyproline was discovered by Fischer. It has as yet been obtained from only a few proteins, but this may be due to the fact that only a few have been examined for its presence. The position of the hydroxyl group has not yet been established.

**Diaminotrihydroxydodecanoic Acid**,  $C_{12}H_{26}N_2O_5$ .—This amino acid was discovered by Fischer and Abderhalden as a product of the hydrolysis of casein. It has thus far been obtained from no other source. It is *lævo*-rotatory and its constitution has not been determined.

#### EXPERIMENTS.

While the ordinary courses in physiological chemistry preclude any extended study of the decomposition products of proteins, the manipulation of a simple decomposition and the subsequent isolation and study of a few of the products most easily and quickly obtained will not be without interest.<sup>2</sup> To this end the student may use the following decomposition

<sup>1</sup> Fischer and Boehner: *Zeit. phys. chem.*, 65, p. 118, 1910.

<sup>2</sup> The procedure here set forth has nothing in common with the procedure by means of which the long line of decomposition products just enumerated are obtained. This latter process is an exceedingly complicated one which is entirely outside the province of any course in physiological chemistry.

procedure: Treat the protein in a large flask with water containing 3-5 per cent of  $H_2SO_4$  and place it on a water-bath until the protein material has been decomposed and there remains a fine, fluffy, insoluble residue. Filter off this residue and neutralize the filtrate with  $Ba(OH)_2$  and  $BaCO_3$ . Filter off the precipitate of  $BaSO_4$  which forms and when certain that the fluid is neutral or faintly acid,<sup>1</sup> concentrate (first on a wire gauze and later on a water-bath) to a syrup. This syrup contains the end-products of the decomposition of the protein, among which are *proteoses*, *peptones*, *tyrosine*, *leucine*, etc. Add 95 per cent alcohol slowly to the warm syrup until no more precipitate forms, stirring continuously with a glass rod. This precipitate consists of proteoses and peptones. Gather the sticky precipitate on the rod or the sides of the dish, and, after warming the solution gently for a few moments, filter it through a filter paper which has not been previously moistened. After dissolving the precipitate of proteoses and peptones in water<sup>2</sup> the solution may be treated according to the method of separation given on page 120.

The leucine and tyrosine, etc., are in solution in the warm alcoholic filtrate. Concentrate this filtrate on the water-bath to a thin syrup, transfer it to a beaker, and allow it to stand over night in a cool place for crystallization. The tyrosine first crystallizes (Fig. 24, page 81), followed later by the formation of characteristic crystals of impure leucine (see Fig. 109, Chapter XX). After examining these crystals under the microscope, strain off the crystalline material through fine muslin, heat it gently in a little water to dissolve the leucine (the tyrosine will be practically insoluble) and filter. Concentrate the filtrate and allow it to stand in a cool place over night for the crude leucine to crystallize. Filter off the crystals and use them in the tests for leucine given on page 91. The crystals of tyrosine remaining on the paper from the first filtration may be used in the tests for tyrosine as given below. If desired, the tyrosine and leucine may be purified by recrystallizing in the usual manner. Habermann has suggested a method of separating leucine and tyrosine by means of glacial acetic acid.

#### EXPERIMENTS ON TYROSINE.

Make the following tests with the tyrosine crystals already prepared or upon some pure tyrosine furnished by the instructor.

1. **Microscopical Examination.**—Place a minute crystal of tyrosine on a slide, add a drop of water, cover with a coverglass, and examine

<sup>1</sup> If the solution is alkaline in reaction at this point, the amino acids will be broken down and ammonia will be evolved.

<sup>2</sup> At this point the aqueous solution of the proteoses and peptones may be filtered to remove any  $BaSO_4$  which may still remain. Tyrosine crystals will also be found here, since it is less soluble than the leucine and may adhere to the proteose-peptone precipitate. Add the crystals of tyrosine to the warm alcohol filtrate.



microscopically. Now run more water under the cover-glass and warm in a bunsen flame until the tyrosine has dissolved. Allow the solution to cool *slowly*, then examine again microscopically, and compare the crystals with those shown in Fig. 24, page 81.

2. **Solubility.**—Try the solubility of very *small amounts* of tyrosine in cold and hot water, cold and hot 95 per cent alcohol, dilute  $\text{NH}_4\text{OH}$ , dilute KOH and dilute HCl.

3. **Sublimation.**—Place a little tyrosine in a *dry* test-tube, heat gently and notice that the material does not sublime. How does this compare with the result of Experiment 3 under Leucine?

4. **Hoffman's Reaction.**—This is the name given to Millon's reaction when employed to detect tyrosine. Add about 3 c.c. of water and a few drops of Millon's reagent to a little tyrosine in a test-tube. Upon dissolving the tyrosine by heat the solution gradually darkens and may assume a dark red color. What group does this test show to be present in tyrosine?

5. **Piria's Test.**—Warm a little tyrosine on a watch glass on a boiling water-bath for 20 minutes with 3–5 drops of conc.  $\text{H}_2\text{SO}_4$ . Tyrosine-sulphuric acid is formed in the process. Cool the solution and wash it into a small beaker with water. Now add  $\text{CaCO}_3$  in substance slowly with stirring, until the reaction of the solution is no longer acid. Filter, concentrate the filtrate, and add to it a few drops (avoid an excess) of very dilute neutral ferric chloride. A purple or violet color, due to the formation of the ferric salt of tyrosine-sulphuric acid, is produced. This is one of the most satisfactory tests for the identification of tyrosine.

6. **Mörner's Test.**—Add about 3 c.c. of Mörner's reagent<sup>1</sup> to a little tyrosine in a test-tube, and *gently* raise the temperature to the boiling-point. A green color results.

#### EXPERIMENTS ON LEUCINE.

Make the following test upon the leucine crystals already prepared or upon some pure leucine furnished by the instructor.

1, 2 and 3. Repeat these experiments according to the directions given under Tyrosine (pages 90 and 91).

<sup>1</sup> Mörner's reagent is prepared by thoroughly mixing 1 volume of formalin, 45 volumes of distilled water, and 55 volumes of concentrated sulphuric acid.

## CHAPTER V.

### PROTEINS : THEIR CLASSIFICATION AND PROPERTIES.

FROM what has already been said in Chapter IV regarding the protein substances it will be recognized that the grouping of the diverse forms of this class of substances in a logical manner is not an easy task. The fats and carbohydrates may be classified upon the fundamental principles of their stereo-chemical relationships, whereas such a system of classification in the case of the proteins is absolutely impossible since, as we have already stated, the molecular structure of these complex substances is unknown. Because of the diversity of standpoint from which the proteins may be viewed, relative to their grouping in the form of a logically classified series, it is obvious that there is an opportunity for the presentation of classifications of a widely divergent character. The fact that there were until recently at least a dozen different classifications which were recognized by various groups of English-speaking investigators emphasizes the difficulties in the way of the individual or individuals who would offer a classification which should merit universal adoption. Realizing the great handicap and disadvantage which the great diversity of the protein classifications was forcing upon the workers in this field, the Chemical and Physiological Societies of England recently drafted a classification which appealed to these groups of scientists as fulfilling all requirements and presented it for the consideration of the American Physiological Society and the American Society of Biological Chemists. The outcome of this has been that there are now only *two* protein classifications which are recognized by English-speaking scientists, one the British Classification, the other the American Classification. These classifications are very similar and doubtless will ultimately be merged into a single classification. In our consideration of the proteins we shall conform in all details to the American Classification. In this connection we will say, however, that we feel that the English Societies have strong grounds for preferring the use of the term *scleroproteins* for albuminoids and *chromoproteins* for hæmoglobins. The two classifications are as follows:

## CLASSIFICATION OF PROTEINS ADOPTED BY THE AMERICAN PHYSIOLOGICAL SOCIETY AND THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

### I. SIMPLE PROTEINS.

Protein substances which yield *only*  $\alpha$ -amino acids or their derivatives on hydrolysis.

(a) **Albumins.**—Soluble in pure water and coagulable by heat, *e. g.*, *ovalbumin*, *serum albumin*, *lactalbumin*, *vegetable albumins*.

(b) **Globulins.**—Insoluble in pure water but soluble in neutral solutions of salts of strong bases with strong acids,<sup>1</sup> *e. g.*, *serum globulin*, *ovoglobulin*, *edestin*, *amandin*, and other *vegetable globulins*.

(c) **Glutelins.**—Simple proteins insoluble in *all neutral solvents*, but readily soluble in very dilute acids and alkalis,<sup>2</sup> *e. g.*, *glutenin*.

(d) **Alcohol-soluble Proteins (Prolamins).**<sup>3</sup>—Simple proteins soluble in 70–80 per cent alcohol, insoluble in water, absolute alcohol, and other neutral solvents,<sup>4</sup> *e. g.*, *zein*, *gliadin*, *hordein*, and *bynin*.

(e) **Albuminoids.**—Simple proteins possessing a similar structure to those already mentioned, but characterized by a pronounced insolubility in all neutral solvents,<sup>5</sup> *e. g.*, *elastin*, *collagen*, *keratin*.

(f) **Histones.**—Soluble in water and insoluble in very dilute ammonia, and, in the absence of ammonium salts, insoluble even in excess of ammonia; yield precipitates with solutions of other proteins and a coagulum on heating which is easily soluble in very dilute acids. On hydrolysis they yield a large number of amino acids among which the basic ones predominate. In short, histones are basic proteins which stand between protamines and true proteins, *e. g.*, *globin*, *thymus histone*, *scombrone*.

(g) **Protamines.**—Simpler polypeptides than the proteins included in the preceding groups. They are soluble in water, uncoagulable by heat, have the property of precipitating aqueous solutions of other proteins, possess strong basic properties and form stable salts with strong mineral acids. They yield comparatively few amino acids, among which the basic ones predominate. They are the *simplest natural proteins*, *e. g.*, *salmine*, *sturine*, *clupeine*, *scombrine*.

<sup>1</sup> The precipitation limits with ammonium sulphate should not be made a basis for distinguishing the albumins from the globulins.

<sup>2</sup> Such substances occur in abundance in the seeds of cereals and doubtless represent a well-defined natural group of simple proteins.

<sup>3</sup> The name *prolamins* has been suggested for these alcohol-soluble proteins by Dr. Thomas B. Osborne (*Science*, 1908, XXVIII, p. 417). It is a very fitting term inasmuch as upon hydrolysis they yield particularly large amounts of *proline* and *ammonia*.

<sup>4</sup> The subclasses defined (*a*, *b*, *c*, *d*), are exemplified by proteins obtained from both plants and animals. The use of appropriate prefixes will suffice to indicate the origin of the compounds, *e. g.*, *ovoglobulin*, *lactalbumin*, etc.

<sup>5</sup> These form the principal organic constituents of the skeletal structure of animals and also their external covering and its appendages. This definition does not provide for gelatin which is, however, an artificial derivative of collagen.

## II. CONJUGATED PROTEINS.

Substances which contain the protein molecule united to some other molecule or molecules otherwise than as a salt.

(a) **Nucleoproteins.**—Compounds of one or more protein molecules with nucleic acid, *e. g.*, *cytoglobulin*, *nucleohistone*.

(b) **Glycoproteins.**—Compounds of the protein molecule with a substance or substances containing a carbohydrate group other than a nucleic acid. *e. g.*, *mucins* and *mucoids* (*osseomucoid*, *tendomucoid*, *seromucoid*, *ichthulin*, *helico*protein).

(c) **Phosphoproteins.**—Compounds of the protein molecule with some, as yet undefined, phosphorus-containing substances other than a nucleic acid or lecithin,<sup>1</sup> *e. g.*, *caseinogen*, *vitellin*.

(d) **Hæmoglobins.**—Compounds of the protein molecule with hæmatin, or some similar substance, *e. g.*, *hæmoglobin*, *hæmocyanin*.

(e) **Lecithoproteins.**—Compounds of the protein molecule with *lecithins*, *e. g.*, *lecithans*, *phosphatides*.

## III. DERIVED PROTEINS.

## I. PRIMARY PROTEIN DERIVATIVES.

Derivatives of the protein molecule apparently formed through hydrolytic changes which involve only slight alteration of the protein molecule.

(a) **Proteans.**—Insoluble products which apparently result from the incipient action of water, very dilute acids or enzymes, *e. g.*, *myosan*, *edestan*.

(b) **Metaproteins.**—Products of the further action of acids and alkalis whereby the molecule is so far altered as to form products soluble in very weak acids and alkalis but insoluble in neutral fluids, *e. g.*, *acid metaprotein* (*acid albuminate*), *alkali metaprotein* (*alkali albuminate*).

(c) **Coagulated Proteins.**—Insoluble products which result from (1) the action of heat on their solutions, or (2) the action of alcohol on the protein.

2. SECONDARY PROTEIN DERIVATIVES.<sup>2</sup>

Products of the further hydrolytic cleavage of the protein molecule.

(a) **Proteoses.**—Soluble in water, non-coagulable by heat, and precipitated by saturating their solutions with ammonium—or zinc sulphate,<sup>3</sup> *e. g.*, *protoproteose*, *deuteroproteose*.

<sup>1</sup> The accumulated chemical evidence distinctly points to the propriety of classifying the phosphoproteins as conjugated compounds, *i. e.*, they are possibly esters of some phosphoric acid or acids and protein.

<sup>2</sup> The term secondary protein derivatives is used because the formation of the primary derivatives usually precedes the formation of the secondary derivatives.

<sup>3</sup> As thus defined, this term does not strictly cover all the protein derivatives commonly called proteoses, *e. g.*, heteroproteose and dysproteose.

(b) **Peptones**.—Soluble in water, non-coagulable by heat, but *not precipitated* by saturating their solutions with ammonium sulphate,<sup>1</sup> *e. g., anti-peptone, amphopeptone.*

(c) **Peptides**.—Definitely characterized combinations of two or more amino acids, the *carboxyl* group of one being united with the *amino* group of the other with the elimination of a molecule of water,<sup>2</sup> *e. g., dipeptides, tripeptides, tetrapeptides, pentapeptides.*

## CLASSIFICATION OF PROTEINS ADOPTED BY THE CHEMICAL AND PHYSIOLOGICAL SOCIETIES OF ENGLAND.

### I. SIMPLE PROTEINS.

1. Protamines, *e. g., salmine, clupeine.*
2. Histones, *e. g., globin, scombrone.*
3. Albumins, *e. g., ovalbumin, serum albumin, vegetable albumins.\**
4. Globulins, *e. g., serum globulin, ovoglobulin, vegetable globulins.*
5. Glutelins, *e. g., glutenin.*
6. Alcohol-soluble proteins, *e. g., zein, gliadin.*
7. Scleroproteins, *e. g., elastin, keratin.*
8. Phosphoproteins, *e. g., caseinogen, vitellin.*

### II. CONJUGATED PROTEINS.

1. Glucoproteins, *e. g., mucins, mucoids.*
2. Nucleoproteins, *e. g., nucleohistone, cytoglobulin.*
3. Chromoproteins, *e. g., hæmoglobin, hæmocyanin.*

### III. PRODUCTS OF PROTEIN HYDROLYSIS.

1. Infraproteins, *e. g., acid infraprotein (acid albuminate), alkali infraprotein (alkali albuminate).*
2. Proteoses, *e. g., protoproteose, heteroproteose, deuteroproteose.*
3. Peptones, *e. g., amphopeptone, anti-peptone.*
4. Polypeptides, *e. g., dipeptides, tripeptides, tetrapeptides.*

## CONSIDERATIONS OF THE VARIOUS CLASSES OF PROTEINS.

### SIMPLE PROTEINS.

The simple proteins are true protein substances which, upon hydrolysis, yield *only*  $\alpha$ -amino acids or their derivatives. "Although

<sup>1</sup> In this group the kyrines may be included. For the present it is believed that it will be helpful to retain this term as defined, reserving the expression *peptide* for the simpler compounds of definite structure, such as dipeptides, etc.

<sup>2</sup> The peptones are undoubtedly peptides or mixtures of peptides, the latter term being at present used to designate those of definite structure.

no means are at present available whereby the chemical individuality of any protein can be established, a number of simple proteins have been isolated from animal and vegetable tissues which have been so well characterized by constancy of ultimate composition and uniformity of physical properties that they may be treated as chemical individuals until further knowledge makes it possible to characterize them more definitely." Under simple proteins we may class albumins, globulins, glutelins, prolamins, albuminoids, histones and protamines.

#### ALBUMINS.

Albumins constitute the first class of simple proteins and may be defined as simple proteins which are coagulable by heat and soluble in pure (salt-free) water. Those of animal origin are not precipitated upon saturating their *neutral* solutions at 30° C. with sodium chloride or magnesium sulphate, but if a saturated solution of this character be acidified with acetic acid the albumin precipitates. All albumins of *animal* origin may be precipitated by saturating their solutions with ammonium sulphate.<sup>1</sup> They may be thrown out of solution by the addition of a sufficient quantity of a mineral acid, whereas a weak acidity produces a slight precipitate which dissolves upon agitating the solution. Metallic salts also possess the property of precipitating albumins, some of the precipitates being soluble in excess of the reagent, whereas others are insoluble in such an excess. Of those proteins which occur native the albumins contain the highest percentage of sulphur, ranging from 1.6 to 2.5 per cent. Some albumins have been obtained in crystalline form, notably egg albumin, serum albumin, and lactalbumin, but the fact that they may be obtained in crystalline form does not necessarily prove them to be chemical individuals.

#### GENERAL COLOR REACTIONS OF PROTEINS.

These color reactions are due to a reaction between some one or more of the constituent radicals or groups of the complex protein molecule and the chemical reagent or reagents used in any given test. Not all proteins contain the same groups and for this reason the various color tests will yield reactions varying in intensity of color according to the nature of the groups contained in the particular protein under examination. Various substances not proteins respond to certain of these color reactions, and it is therefore essential to submit the material under examination to several tests before concluding definitely regarding its nature.

<sup>1</sup> In this connection, Osborne's observation that there are certain *vegetable* albumins which are precipitated by saturating their solutions with sodium chloride or magnesium sulphate or by half-saturating with ammonium sulphate, is of interest.

## TECHNIC OF THE COLOR REACTIONS.

1. **Millon's Reaction.**—To 5 c.c. of a dilute solution of egg albumin in a test-tube add a few drops of Millon's reagent. A white precipitate forms which turns red when heated. This test is a particularly satisfactory one for use on *solid* proteins, in which case the reagent is added directly to the solid substance and heat applied, which causes the substance to assume a red color. Such proteins as are not precipitated by mineral acids, for example certain of the proteoses and peptones, yield a *red solution* instead of a red precipitate.

The reaction is due to the presence of the *hydroxy-phenyl group*,  $-C_6H_4OH$ , in the protein molecule and certain non-proteins such as tyrosine, phenol (carbolic acid) and thymol also respond to the reaction. Inasmuch as the tyrosine grouping is the only hydroxyphenyl grouping which has definitely been proven to be present in the protein molecule it is evident that protein substances respond to Millon's reaction because of the presence of this tyrosine complex. The test is not a very satisfactory one for use in solutions containing inorganic salts in large amount, since the mercury of the Millon's reagent<sup>1</sup> is thus precipitated and the reagent rendered inert. This reagent is therefore never used for the detection of protein material in the urine.

2. **Xanthoproteic Reaction.**—To 2-3 c.c. of egg albumin solution in a test-tube add concentrated nitric acid. A white precipitate forms, which upon heating turns yellow and finally dissolves, imparting to the solution a yellow color. *Cool the solution* and carefully add ammonium hydroxide, potassium hydroxide, or sodium hydroxide *in excess*. Note that the yellow color deepens into an orange. This reaction is due to the presence in the protein molecule of the *phenyl group*, with which the nitric acid forms certain nitro modifications. The particular complexes of the protein molecule which are of especial importance in this connection are those of tyrosine, phenylalanine, and tryptophane. The test is not a satisfactory one for use in urinary examination because of the color of the end-reaction.

3. **Adamkiewicz Reaction.**—Thoroughly mix 1 volume of concentrated sulphuric acid and 2 volumes of acetic acid in a test-tube, add a few drops of egg albumin solution and heat gently. A reddish-violet color is produced. Gelatin does not respond to this test. This reaction shows the presence of the *tryptophane group* (see next experiment). The test depends upon the presence of glyoxylic acid,  $CHO-COOH + H_2O$  or  $CH(OH)_2COOH$ , in the reagents. This is shown

<sup>1</sup>Millon's reagent consists of mercury dissolved in nitric acid containing some nitrous acid. It is prepared by digesting one part (by weight) of mercury with two parts (by weight) of  $HNO_3$  (sp. gr. 1.42) and diluting the resulting solution with two volumes of water.

by the failure to secure a positive reaction when acetic acid free from glyoxylic acid is used.

Rosenheim has recently advanced the view that the reaction may be due to the presence of oxidizing agents such as nitrous acid and ferric salts in the sulphuric acid.

4. **Hopkins-Cole Reaction.**<sup>1</sup>—Place 1–2 c.c. of egg albumin solution and 3 c.c. of glyoxylic acid,  $\text{CHO.COOH} + \text{H}_2\text{O}$  or  $\text{CH}(\text{OH})_2\text{-COOH}$ , solution (Hopkins-Cole reagent<sup>2</sup>) in a test-tube and mix thoroughly. In a second tube place 5 c.c. of concentrated sulphuric acid. Incline the tube containing the sulphuric acid and by means of a pipette allow the albumin-glyoxylic acid solution to flow *carefully* down the side. When stratified in this manner a reddish-violet color forms at the zone of contact of the two fluids. This color is due to the presence of the *tryptophane group*. Gelatin does not respond to this test. For formula for tryptophane see page 82.

Benedict<sup>3</sup> has recently suggested a new reagent for use in carrying out the Hopkins-Cole reaction.<sup>4</sup>

5. **Biuret Test.**—To 2–3 c.c. of egg albumin solution in a test-tube add an equal volume of concentrated potassium hydroxide solution, mix thoroughly, and add slowly a very dilute (2–5 drops in a test-tube of water) copper sulphate solution until a purplish-violet or pinkish-violet color is produced. The depth of the color depends upon the nature of the protein; proteoses, and peptones giving a decided pink, while the color produced with gelatin is not far removed from a blue. This reaction is given by those substances which contain *two amino groups* in their molecule, these groups either being joined directly together or through a single atom of nitrogen or carbon. The amino groups mentioned must either be two  $\text{CONH}_2$  groups or one  $\text{CONH}_2$  group and one  $\text{CSNH}_2$ ,  $\text{C}(\text{NH})\text{-NH}_2$  or  $\text{CH}_2\text{NH}_2$  group. It follows from this fact that substances which are non-protein in character but which contain the necessary groups will respond to the biuret test. As examples of such substances may be cited *oxamide*,

<sup>1</sup> Hopkins and Cole: *Journal of Physiology*, 27, 418, 1902.

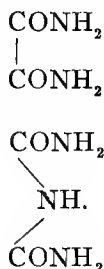
<sup>2</sup> Hopkins-Cole reagent is prepared as follows: To one liter of a saturated solution of oxalic acid add 60 grams of sodium amalgam and allow the mixture to stand until the evolution of gas ceases. Filter and dilute with 2–3 volumes of water.

<sup>3</sup> Benedict: *Journal of Biological Chemistry*, 6, 51, 1909.

<sup>4</sup> Benedict's modified Hopkins-Cole reagent is prepared as follows: Ten grams of powdered magnesium are placed in a large Erlenmeyer flask and shaken up with enough distilled water to liberally cover the magnesium. Two hundred and fifty c.c. of a cold, saturated solution of oxalic acid is now added slowly. The reaction proceeds very rapidly and with the liberation of much heat, so that the flask should be cooled under running water during the addition of the acid. The contents of the flask are shaken after the addition of the last portion of the acid and then poured upon a filter, to remove the insoluble magnesium oxalate. A little wash water is poured through the filter, the filtrate acidified with acetic acid to prevent the partial precipitation of the magnesium on long standing, and made up to a liter with distilled water. This solution contains only the magnesium salt of glyoxylic acid.

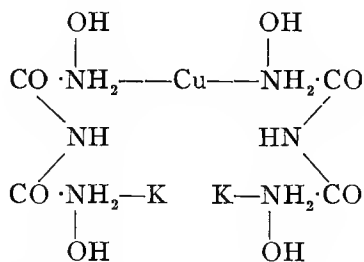


and *biuret*,



The test derives its name from the fact that this latter substance which is formed on heating urea to 180° C. (see page 286), will respond to the test. Protein material responds positively since there are two CONH<sub>2</sub> groups in the protein molecule.

According to Schiff the end-reaction of the biuret test is dependent upon the formation of a copper-potassium-biuret compound (cupri-potassium biuret or biuret potassium cupric hydroxide). This substance was obtained by Schiff in the form of long red needles. It has the following formula:



6. *Gies's Biuret Reagent*.<sup>1</sup>—Gies has recently devised a reagent for use in the biuret test. This reagent consists of 10 per cent KOH solution, to which enough 3 per cent CuSO<sub>4</sub> solution has been added to impart a slight though distinct blue color to the clear liquid. The CuSO<sub>4</sub> should be added drop by drop with thorough shaking after each addition. This reagent is of material assistance in performing the biuret test.

7. *Biuret Paper of Kantor and Gies*.—According to Kantor and Gies<sup>2</sup> when filter paper is immersed in the above reagent and subsequently dried it forms a very satisfactory “biuret paper” which may be used in a manner analogous to indicator papers. Moist papers may be used in the examination of powders which are neutral or alkaline in reaction. In preparing the “biuret paper” if the filter paper is left for a sufficient length of time in the reagent all traces of the copper sulphate will be removed from the solution.

<sup>1</sup> Gies: Proceedings of Society of Biological Chemists, *Journal of Biological Chemistry*, 7, 60, 1910.

<sup>2</sup> Kantor and Gies: *Proc. Soc. Biol. Chem.*, p. 11, 1910.

8. **Posner's Modification of the Biuret Test.**—This test is particularly satisfactory for use on *dilute* protein solutions, and is carried out as follows: To some dilute egg albumin in a test-tube add one-half its volume of potassium hydroxide solution. Now hold the tube in an inclined position and allow some very dilute copper sulphate solution, made as suggested on page 98 (5), to flow down the side, being especially careful to prevent the fluids from mixing. At the juncture of the two solutions the typical end-reaction of the biuret test should appear as a colored zone (see Biuret Test, page 98).

9. *Testing Colored Solutions by Biuret Test.*—If the color of the solution is such as to interfere with the end-reaction of the biuret test proceed as follows: Make the solution strongly alkaline with potassium hydroxide and add a solution of copper sulphate. Shake up the mixture with alcohol and if protein is present the alcohol will assume the typical biuret coloration. This procedure is not applicable in case the pigment of the original solution is soluble in alcohol. Excess of the copper salt need not be avoided in this test.

10. **Liebermann's Reaction.**—Add about 10 drops of *concentrated* egg albumin solution (or a little dry egg albumin) to about 5 c.c. of concentrated HCl in a test-tube. Boil the mixture until a pinkish-violet color results. This color was originally supposed to indicate the presence of a carbohydrate group in the protein molecule, the furfural formed through the action of the acid upon the protein reacting with the *hydroxy-phenyl group* of the protein producing the pinkish-violet color. It is now considered *uncertain* whether the carbohydrate group enters into the reaction. Cole has called attention to the fact that a *blue* color results if protein material which has been boiled with alcohol and subsequently *washed with ether* be used in making the test. He believes the blue color to be due to an interaction between the glyoxylic acid, which was present as an impurity in the ether used in washing the protein, and the *tryptophane group* of the protein molecule which was split off through the action of the acid.

11. **Acree-Rosenheim Formaldehyde Reaction.**—Add a few drops of a dilute (1:5000) solution of formaldehyde to 2–3 c.c. of egg albumin solution in a test-tube. Mix thoroughly and after 2–3 minutes carefully introduce a little concentrated sulphuric acid into the tube in such a manner that the two solutions do not mix. A violet zone will be observed at the point of juncture of the two solutions especially if the mixture is slightly agitated. This color probably results through the union of the protein and the formaldehyde. If the sulphuric acid is added to the protein *before* the formaldehyde is added the typical end-reaction is not obtained. So far as is known this is a specific test for proteins.

The reaction cannot be applied satisfactorily with concentrated formaldehyde.

Rosenheim claims the reaction is due to the presence of oxidizing material in the sulphuric acid and that when pure sulphuric acid is used no reaction is obtained. He advises the use of a slight amount of an oxidizing agent, *e. g.*, ferric chloride or potassium nitrate (0.005 gram per 100 c.c. of sulphuric acid) in order to facilitate the reaction. Rosenheim further states that proteins respond to the formaldehyde reaction because of the presence of the *tryptophane group*, a statement which Acree does not accept as proven.

12. **Bardach's Reaction.**<sup>1</sup>—This is one of the most recent tests which have been described for the detection of protein material. The test depends upon the property possessed by protein substances of preventing the formation of typical iodoform crystals through the interaction of an alkaline acetone solution with iodopotassium iodide. Instead of the typical hexagonal plates or stellar formations of iodoform there are produced, under the conditions of the test, *fine yellow needles* which are apparently some iodine compound other than iodoform. The technic of the test is as follows: Place about 5 c.c. of the protein solution<sup>2</sup> under examination in a test-tube, add 2–3 drops of a 0.5 per cent solution of acetone and sufficient Lugol's solution<sup>3</sup> to supply a moderate excess of iodine and produce a red-brown coloration. (The amount of Lugol's solution necessary will depend upon the content of protein, sugar, and other iodine-reacting substances in the solution under examination and may vary from one drop to several cubic centimeters.) Add an excess (ordinarily about 3 c.c.) of concentrated ammonium hydroxide and thoroughly mix the solution. Place the tube in the test-tube rack, examine the contents at intervals of five minutes, and when it is evident that crystals have formed, place a drop of the mixture upon a microscopic slide, put a coverglass in position, and examine the mixture under the microscope. The formation of *canary yellow crystals* indicates the presence of protein material in the solution examined. The crystals are ordinarily needle-like in appearance and show a tendency to assume rosette or bundle-like formations, but under certain conditions they may show knobbed (nail-like) and branching variations.

If a moderate excess of iodine is used in making the test, a black precipitate of iodonitro compounds is at once formed upon the addition of the ammonium hydroxide, and yellow needles are subsequently deposited upon it. In case just the proper amount of iodine is used, the solution

<sup>1</sup> Bardach: *Zeitschrift für Physiologische Chemie*, 54, 355, 1908; also Seaman and Gies: *Proceedings of the Society for Experimental Biology and Medicine*, 5, 125, 1908.

<sup>2</sup> The solution should not contain more than 5 per cent of protein material.

<sup>3</sup> Dissolve 4 grams of iodine and 6 grams of potassium iodide in 100 c.c. of distilled water.

soon assumes a yellow color and the black precipitate formed upon the addition of the ammonium hydroxide is gradually transformed more or less completely into the yellow crystals. In either case the needles ordinarily form within an hour, and frequently in a much shorter time. If too great an excess of iodine is employed the heavy black precipitate may obscure or even prevent the reaction. The presence of insufficient iodine or excess protein may likewise prevent the reaction. In tests in which a concentrated protein solution and an excess of iodine are used, the addition of ammonium hydroxide immediately produces a grayish-green precipitate. In such instances, if the proportions are favorable, and the mixture be stirred with a glass rod for a few minutes, the precipitate is gradually transformed into the crystals before mentioned.

It is probable that all soluble proteins will respond to Bardach's reaction, but the relative delicacy of the reaction as well as the value of the test as compared with other protein tests remain to be determined. The only disturbing factor noted thus far is the presence of earthy phosphates in the solution under examination.

### PRECIPITATION REACTIONS AND OTHER PROTEIN TESTS.

There are three forms in which proteins may be precipitated, *i. e.*, *unaltered*, as an *albuminate*, and as an *insoluble salt*. An instance of the precipitation in a *native* or *unaltered* condition is seen in the so-called *salting-out* experiments. Various salts, notably  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{MgSO}_4$ ,  $\text{Na}_2\text{SO}_4$  and  $\text{NaCl}$  possess the power when added in *solid form* to certain definite protein solutions, of rendering the menstruum incapable of holding the protein in solution, thereby causing the protein to be precipitated or *salted-out* to use the common term. Mineral acids and alcohol also precipitate proteins unaltered. In the case of concentrated acids the protein is dissolved in the presence of an excess of acid with the formation of a protein salt. Proteins are precipitated as *albuminates* (*protein salts*) when treated with certain metallic salts, and precipitated as *insoluble salts* when weak organic acids such as certain of the alkaloidal reagents are added to their solutions.

If certain acids (picric, tungstic, phosphomolybdic, tannic, or chromic) be added to a *neutral* albumin solution a precipitate of a *protein salt* occurs. If, however, the salts of these acids be added no precipitate occurs. The addition of a small amount of acid, as acetic acid, to such a solution will cause a precipitate to form.<sup>1</sup>

The effect of the addition of the salts of the heavy metals is in the first instance to cause a precipitation of the protein. In many cases, however,

<sup>1</sup> Mathews: *Amer. Jour. of Physiology*, 1, 445, 1898.

the addition of an excess of such salts causes the solution of the precipitate while a further excess may cause a reprecipitation. The precipitate which is *first* formed in a protein solution by the addition of the salts of the heavy metals may be redissolved not only by an excess of such salts but by an excess of protein as well.<sup>1</sup>

It is generally stated that globulins are precipitated from their solutions upon *half* saturation with ammonium sulphate and that albumins are precipitated upon *complete* saturation by this salt. Comparatively few exceptions were found to this rule until proteins of *vegetable* origin came to be more extensively studied. These studies, furthered especially by Osborne and associates, have demonstrated very clearly that the characterization of a globulin as a protein which is precipitated by *half* saturation with ammonium sulphate, can no longer hold. Certain vegetable globulins have been isolated which are not precipitated by this salt until a concentration is reached *greater than that secured by half-saturation*. As an example of an albumin which does not conform to the definition of an albumin as regards its precipitation by ammonium sulphate, may be mentioned the *leucosin* of the wheat germ which is precipitated from its solution upon *half-saturation* with ammonium sulphate. The limits of precipitation by ammonium sulphate, therefore, do not furnish a sufficiently accurate basis for the differentiation of globulins from albumins. It has further been determined that a given protein which is precipitable by ammonium sulphate cannot be "salted-out" by the same concentration of the salt under all conditions.

## EXPERIMENTS.

1. **Influence of Concentrated Mineral Acids, Alkalis and Organic Acids.**—Prepare five test-tubes each containing 5 c.c. of concentrated egg albumin solution. To the first add concentrated  $H_2SO_4$ , drop by drop, until an excess of the acid has been added. Note any changes which may occur in the solution. Allow the tube to stand for 24 hours and at the end of that period observe any alteration which may have taken place. Heat the tube and note any further change which may occur. Repeat the experiment in the four remaining tubes with concentrated hydrochloric acid, concentrated nitric acid, concentrated potassium hydroxide and acetic acid. How do strong mineral acids, strong alkalis, and strong organic acids differ in their action toward protein solutions?

2. **Precipitation by Metallic Salts.**—Prepare four tubes each containing 2–3 c.c. of dilute egg albumin solution. To the first add *mercuric*

<sup>1</sup> Pauli: *Hofmeister's Beiträge*, 6, 233, 1904–5. Robertson: *Ergebnisse der Physiologie*, 10, 290, 1910.

*chloride, drop by drop*, until an excess of the reagent has been added, noting any changes which may occur. Repeat the experiment with *lead acetate, silver nitrate, copper sulphate, ferric chloride, and barium chloride*.

Egg albumin is used as an antidote for lead or mercury poisoning. Why?

3. **Precipitation by Alkaloidal Reagents.**—Prepare six tubes each containing 2–3 c.c. of dilute egg albumin solution. To the first add *picric acid* drop by drop until an excess of the reagent has been added, noting any changes which may occur. Repeat the experiment with *trichloroacetic acid, tannic acid, phosphotungstic acid, phosphomolybdic acid, and potassio-mercuric iodide*. Acidify with hydrochloric acid before testing with the three last reagents.

4. **Heller's Ring Test.**—Place 5 c.c. of concentrated nitric acid in a test-tube, incline the tube, and by means of a pipette allow the dilute albumin solution to flow *slowly* down the side. The liquids should stratify with the formation of a white zone of precipitated albumin at the point of juncture. This is a very delicate test and is further discussed on p. 333.

An apparatus called the *albumoscope* or *horismascope* has been devised for use in the tests of this character and has met with considerable favor. The method of using the albumoscope is described below.

*Use of the Albumoscope.*—This instrument is intended to facilitate the making of “ring” tests such as Heller's and Roberts'. In making a test about 5 c.c. of the solution under examination is first introduced into the apparatus through the larger arm and the reagent used in the particular test is then introduced through the capillary arm and allowed to flow down underneath the solution under examination. If a reasonable amount of care is taken there is no possibility of mixing the two solutions and a definitely defined white “ring” is easily obtained at the zone of contact.

5. **Roberts' Ring Test.**—Place 5 c.c. of Roberts' reagent<sup>1</sup> in a test-tube, incline the tube, and by means of a pipette allow the albumin solution to flow *slowly* down the side. The liquids should stratify with the formation of a *white* zone of precipitated albumin at the point of juncture. This test is a modification of Heller's ring test and is rather more satisfactory. The albumoscope may also be used in making this test. (See page 334.)

6. **Spiegler's Ring Test.**—Place 5 c.c. of Spiegler's reagent<sup>2</sup> in a test-

<sup>1</sup> Roberts' reagent is composed of 1 volume of concentrated HNO<sub>3</sub> and 5 volumes of a saturated solution of MgSO<sub>4</sub>.

<sup>2</sup> Spiegler's reagent has the following composition:

Tartaric acid.....	20 grams.
Mercuric chloride.....	40 grams.
Glycerol.....	100 grams.
Distilled water.....	1000 grams.

tube, incline the tube, and by means of a pipette allow 5 c.c. of albumin solution, acidified with acetic acid, to flow slowly down the side. A white zone will form at the point of contact. This is an exceedingly delicate test, in fact too delicate for ordinary clinical purposes, since it serves to detect albumin when present in the merest trace (1:250,000). This test is further discussed on page 335.

7. **Jolles' Reaction.**—Shake 5 c.c. of albumin solution with 1 c.c. of 30 per cent acetic acid and 4 c.c. of Jolles' reagent<sup>1</sup> in a test-tube. A *white* precipitate of albumin should form. Care should be taken to use the correct amount of acetic acid. For further discussion of the test see page 335.

8. **Tanret's Test.**—To 5 c.c. of albumin solution in a test-tube add Tanret's reagent,<sup>2</sup> drop by drop, until a turbidity or precipitate forms. This is an exceedingly delicate test. Sometimes the albumin solution is stratified upon the reagent as in Heller's or Roberts' ring tests. In urine examination it is claimed by Repiton that the presence of urates lowers the delicacy of the test. Tanret has, however, very recently made a statement to the effect that the removal of urates is not necessary inasmuch as the urate precipitate will disappear on warming and the albumin precipitate will not. He says, however, that mucin interferes with the delicacy of his test and should be removed by acidification with acetic acid and filtration before testing for albumin.

9. **Sodium Chloride and Acetic Acid Test.**—Mix 2 volumes of albumin solution and 1 volume of a saturated solution of sodium chloride in a test-tube, acidify with acetic acid, and heat to boiling. The production of a cloudiness or the formation of a precipitate indicates the presence of albumin.

10. **Potassium Iodide Test.**—Stratify a dilute albumin solution upon a solution of potassium iodide made slightly acid with acetic acid. In the presence of 0.01–0.02 per cent of albumin a white ring forms immediately. If the test be allowed to stand two minutes after the stratification it will serve to detect 0.005 per cent of albumin.

11. **Acetic Acid and Potassium Ferrocyanide Test.**—To 5 c.c. of dilute egg albumin solution in a test-tube add 5–10 drops of acetic acid. Mix well, and add potassium ferrocyanide, *drop by drop*, until a precipitate forms. This test is very delicate.

<sup>1</sup> Jolles' reagent has the following composition:

Succinic acid.....	40 grams.
Mercuric chloride.....	20 grams.
Sodium chloride.....	20 grams.
Distilled water.....	1000 grams.

<sup>2</sup> Tanret's reagent is prepared as follows: Dissolve 1.35 gram of mercuric chloride in 25 c.c. of water, add to this solution 3.32 grams of potassium iodide dissolved in 25 c.c. of water, then make the total solution up to 60 c.c. with water and add 20 c.c. of glacial acetic acid to the combined solutions.

Schmiedl claims that a precipitate of  $\text{Fe}(\text{Cn})_6\text{K}_2\text{Zn}$  or  $\text{Fe}(\text{Cn})_6\text{Zn}_2$ , is formed when solutions containing zinc are subjected to this test, and that this precipitate resembles the precipitate secured with protein solutions. In the case of human urine a reaction was obtained when 0.000022 gram of zinc per cubic centimeter was present. Schmiedl further found that the urine collected from rabbits housed in zinc-lined cages possessed a zinc content which was sufficient to yield a ready response to the test. Zinc is the only interfering substance so far reported.

**12. Salting-out Experiments.**—(a) To 25 c.c. of egg albumin solution in a small beaker add *solid* ammonium sulphate to the point of saturation, keeping the temperature of the solution below 40° C. Filter, test the precipitate by Millon's reaction and the filtrate by the biuret test. What are your conclusions? (b) Repeat the above experiment making the saturation with *solid* sodium chloride. How does this result differ from the result of the saturation with ammonium sulphate? Add 2–3 drops of acetic acid. What occurs? All proteins *except peptones* are precipitated by saturating their solutions with ammonium sulphate. *Globulins* are the only proteins precipitated by saturating with sodium chloride (see *Globulins*, page 109), unless the saturated solution is subsequently acidified, in which event all proteins *except peptones* are precipitated.

Soaps may be salted-out in a similar manner (see p. 145).

**13. Coagulation or Boiling Test.**—Heat 25 c.c. of dilute egg albumin solution to the boiling-point in a small evaporating dish. The albumin coagulates. Complete coagulation may be obtained by acidifying the solution with 3–5 drops of acetic acid<sup>1</sup> *at the boiling-point*. Test the coagulum by Millon's reaction. The acid is added to neutralize any possible alkalinity of the solution, to dissolve any substances which are not albumin and to facilitate coagulation (see further discussion on pages 117 and 335).

**14. Coagulation Temperature.**—Prepare 4 test-tubes each containing 5 c.c. of *neutral* egg albumin solution. To the first add 1 drop of 0.2 per cent hydrochloric acid, to the second add 1 drop of 0.5 per cent sodium carbonate solution, to the third add 1 drop of 10 per cent sodium chloride solution and leave the fourth neutral in reaction. Partly fill a beaker of medium size with water and place it within a second larger beaker which also contains water, the two vessels being separated by pieces of cork. Fasten the four test-tubes compactly together by means of a rubber band, lower them into the water of the inner beaker and suspend them, by means of a clamp attached to one of the tubes, in such a

<sup>1</sup> Nitric acid is often used in place of acetic acid in this test. In case nitric acid is used, ordinarily 1–2 drops is sufficient.



manner that the albumin solutions shall be midway between the upper and lower surfaces of the water. In one of the tubes place a thermometer with its bulb entirely beneath the surface of the albumin solution (Fig. 33). Gently heat the water in the beakers, noting carefully any changes which may occur in the albumin solutions and record the exact temperature at which these changes occur. The first appearance of an *opacity* in an albumin solution indicates the commencement of coagulation and the temperature at which this occurs should be recorded as the *coagulation temperature* for that particular albumin solution.

What is the order in which the four solutions coagulate?

Repeat the experiment, adding to the first tube 1 drop of acetic acid, to the second 1 drop of concentrated potassium hydroxide solution, to the third 2 drops of a 10 per cent sodium chloride solution and leave the fourth neutral as before.

What is the order of coagulation here? Why?

15. **Precipitation by Alcohol.**—Prepare 3 test-tubes each containing about 10 c.c. of 95 per cent alcohol. To the first add one drop of 0.2 per cent hydrochloric acid, to the second one drop of potassium hydroxide solution and leave the third neutral in reaction. Add to each tube a few drops of egg albumin solution and note the results. What do you conclude from this experiment? Alcohol precipitates proteins unaltered, but if allowed to remain under alcohol the protein is transformed. The “fixing” of tissues for histological examination by means of alcohol is an illustration of the application of this transformation produced by alcohol. It apparently is a process of dehydration.

16. **Preparation of Powdered Egg Albumin.**—This may be prepared as follows: Ordinary egg-white finely divided by means of scissors or a beater is treated with four volumes of water and filtered. The filtrate is evaporated on a water-bath at about 50° C. and the residue powdered in a mortar.

17. **Tests on Powdered Egg Albumin.**—With powdered albumin

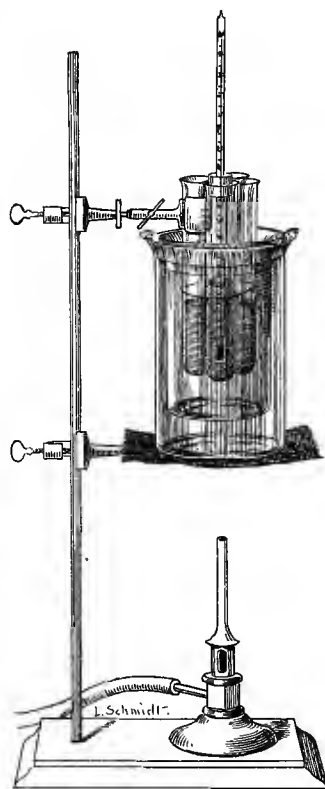


FIG. 33.—COAGULATION TEMPERATURE APPARATUS.

prepared as described above (by yourself or furnished by the instructor), try the following tests:

(a) *Solubility.*

(b) *Millon's Reaction.*

(c) *Hopkins-Cole Reaction.*—When used to detect the presence of protein in solid form this reaction should be conducted as follows: Place 5 c.c. of concentrated sulphuric acid in a test-tube and add carefully, by means of a pipette, 3–5 c.c. of Hopkins-Cole reagent. Introduce a small amount of the solid substance to be tested, agitate the tube slightly, and note that the suspended pieces assume a reddish-violet color, which is the characteristic end-reaction of the Hopkins-Cole test; later the solution will also assume the reddish-violet color.

(d) *Composition Test.*—Heat some of the powder in a test-tube in which is suspended a strip of moistened red litmus paper and across the mouth of which is placed a piece of filter paper moistened with lead acetate solution. As the powder is heated it chars, indicating the presence of *carbon*; the fumes of ammonia are evolved, turning the red litmus paper blue and indicating the presence of *nitrogen* and *hydrogen*; the lead acetate paper is blackened, indicating the presence of sulphur, and the deposition of moisture on the side of the tube indicates the presence of *hydrogen*.

(e) Immerse a dry test-tube containing a little powdered egg albumin in boiling water for a few moments. Remove and test the solubility of the albumin according to the directions given under (a) above. It is still soluble. Why has it not been coagulated? Repeat the above experiments with powdered serum albumin and see how the results compare with those just obtained.

## SULPHUR IN PROTEIN.

Sulphur is believed to be present in two different forms in the protein molecule. The first form, which is present in greatest amount, is that loosely combined with carbon and hydrogen. Sulphur in this form is variously termed *unoxidized*, *loosely combined*, *mercaptan*, and *lead-blackening* sulphur. The second form is combined in a more stable manner with carbon and oxygen and is known as *oxidized* or *acid* sulphur. The protamines are the only class of sulphur-free proteins.

### TESTS FOR SULPHUR.

1. **Tests for Loosely Combined Sulphur.**—(a) To equal volumes of KOH and egg albumin solutions in a test-tube add 1–2 drops of lead acetate solution and boil the mixture. Loosely combined sulphur is

indicated by a darkening of the solution, the color deepening into a black if sufficient sulphur is present. Add hydrochloric acid and note the characteristic odor evolved from the solution. Write the reactions for this test. (b) Place equal volumes of KOH and egg albumin solutions in a test-tube and boil the mixture vigorously. Cool, make acid with glacial acetic acid and add 1-2 drops of lead acetate. A darkening indicates the presence of loosely combined sulphur.

2. **Test for Total Sulphur (Loosely Combined and Oxidized).**—Place the substance to be examined (powdered egg albumin) in a small porcelain crucible, add a suitable amount of solid fusion mixture (potassium hydroxide and potassium nitrate mixed in the proportion 5:1) and heat carefully until a colorless mixture results. (Sodium peroxide may be used in place of this fusion mixture if desired.) Cool, dissolve the cake in a little warm water and filter. Acidify the filtrate with hydrochloric acid, heat it to the boiling-point and add a small amount of barium chloride solution. A white precipitate forms if sulphur is present. What is this precipitate?

#### GLOBULINS.

Globulins are simple proteins especially predominant in the vegetable kingdom. They are closely related to the albumins and in common with them give all the ordinary protein tests. Globulins differ from the albumins in being insoluble in pure (salt-free) water. They are, however, soluble in neutral solutions of salts of strong bases with strong acids. Most globulins are precipitated from their solutions by saturation with solid sodium chloride or magnesium sulphate. As a class they are much less stable than the albumins, a fact shown by the increasing difficulty with which a globulin dissolves during the course of successive reprecipitations.

We have used an albumin of animal origin (egg albumin) for all the protein tests thus far, whereas the globulin to be studied will be prepared from a vegetable source. There being no essential difference between animal and vegetable proteins, the vegetable globulin we shall study may be taken as a true type of all globulins, both animal and vegetable.

#### EXPERIMENTS ON GLOBULIN.

**Preparation of the Globulin.**—Extract 20-30 grams (a handful) of crushed hemp seed with a 5 per cent solution of sodium chloride for one-half hour at 60° C. Filter while hot through a paper moistened with 5 per cent sodium chloride solution. Place the filtrate in the water-bath at 60° C. and allow it to stand for 24 hours in order that the globulin

may crystallize slowly. In case the filtrate is *cloudy* it should be warmed to  $60^{\circ}$  C. in order to produce a *clear* solution. The globulin is soluble in *hot* 5 per cent sodium chloride solution and is thus extracted from the

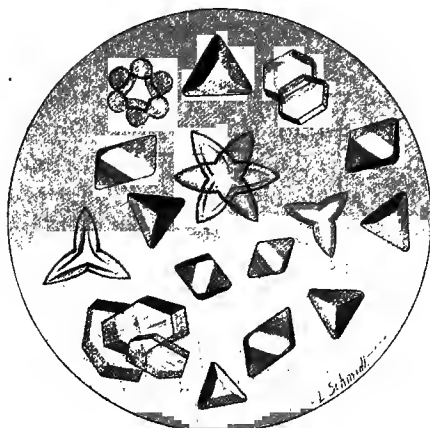


FIG. 34.—EDESTIN.

hemp seed, but upon cooling this solution much of the globulin separates in crystalline form. This particular globulin is called *edestin*. It crystallizes in several different forms, chiefly octahedra (see Fig. 34, above). (The crystalline form of *excelsin*, a protein obtained from the Brazil nut, is shown in Fig. 35, below. This vegetable protein crystallizes in the

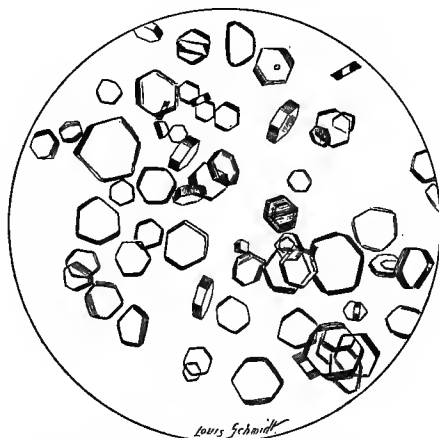


FIG. 35.—EXCELSIN, THE PROTEIN OF THE BRAZIL NUT.  
(Drawn from crystals furnished by Dr. Thomas B. Osborne, New Haven, Conn.)

form of hexagonal plates.) Filter off the edestin and make the following tests on the crystalline body and on the filtrate which still contains some of the extracted globulin.

TESTS ON CRYSTALLIZED EDESTIN.—(1) *Microscopical examination* (see Fig. 34, p. 110).

(2) *Solubility*.—Try the solubility in the ordinary solvents (see page 27). Keep these solubilities in mind for comparison with those of edestan, to be made later (see page 115).

(3) *Millon's Reaction*.

(4) *Coagulation Test*.—Place a small amount of the globulin in a test-tube, add a little water and boil. Now add dilute hydrochloric acid and note that the protein no longer dissolves. It has been coagulated.

(5) Dissolve the remainder of the edestin in 0.2 per cent hydrochloric acid and preserve this acid solution for use in the experiments on proteans (see page 115).

TESTS ON EDESTIN FILTRATE.—(1) *Influence of Protein Precipitants*.—Try a few protein precipitants such as *nitric acid*, *tannic acid*, *picric acid*, and *mercuric chloride*.

(2) *Biuret Test*.

(3) *Coagulation Test*.—Boil some of the filtrate in a test-tube. What happens?

(4) *Saturation with Sodium Chloride*.—Saturate some of the filtrate with *solid* sodium chloride. How does this result differ from that obtained upon saturating egg albumin solution with *solid* sodium chloride?

(5) *Precipitation by Dilution*.—Dilute some of the filtrate with 10–15 volumes of water. Why does the globulin precipitate?

### Glutelins.

It has been repeatedly shown, particularly by Osborne, that after extracting the seeds of cereals with water, neutral salt solution, and strong alcohol, there still remains a residue which contains protein material which may be extracted by very dilute acid or alkali. These proteins which are insoluble in all neutral solvents, but readily soluble in very dilute acids and alkalis are called *glutelins*. The only member of the group which has yet received a name, is the *glutenin* of wheat, a protein which constitutes nearly 50 per cent of the gluten. It is not definitely known whether glutelins occur as constituents of all seeds.

### Prolamins (Alcohol-soluble Proteins).

The term prolamins has been proposed by Osborne, for the group of proteins formerly termed "alcohol-soluble proteins." The name is very appropriate inasmuch as these proteins yield, upon hydrolysis, especially large amounts of *proline* and *ammonia*. The prolamins are simple proteins which are insoluble in water, *absolute* alcohol and other

neutral solvents, but are soluble in 70 to 80 per cent alcohol and in dilute acids and alkalis. They occur widely distributed, particularly in the vegetable kingdom. The only prolamins yet described are the *zein* of maize, the *hordein* of barley, the *gliadin* of wheat and rye, and the *bymin* of malt. They yield relatively large amounts of glutamic acid on hydrolysis but *no lysin*. The largest percentage of glutamic acid (43.66 per cent) ever obtained as a decomposition product of a protein substance has very recently been obtained by Osborne & Guest from the hydrolysis of the prolamins *gliadin*.<sup>1</sup> This yield of glutamic acid is also the largest amount of any single decomposition product yet obtained from any protein *except protamines*.

### Albuminoids. (Scleroproteins.)

The albuminoids yield similar hydrolytic products to those obtained from the other simple proteins already considered, thus indicating that they possess essentially the same chemical structure. They differ from all other proteins, whether simple, conjugated, or derived, in that they are insoluble in all neutral solvents. The albuminoids include "the principal organic constituents of the skeletal structure of animals as well as their external covering and its appendages. Some of the principal albuminoids are *keratin*, *elastin*, *collagen*, *reticulin*, *spongin*, and *fibroin*. Gelatin cannot be classed as an albuminoid although it is a transformation product of collagen. The various albuminoids differ from each other in certain fundamental characteristics which will be considered in detail under Epithelial and Connective Tissue (see Chapter XIV, p. 245).

### CONJUGATED PROTEINS.

Conjugated proteins consist of a protein molecule united to some other molecule or molecules otherwise than as a salt. We have *glycoproteins*, *nucleoproteins*, *hæmoglobins* (chromoproteins), *phosphoproteins* and *lecithoproteins* as the five classes of conjugated proteins.

Glycoproteins may be considered as compounds of the protein molecule with a substance or substances containing a carbohydrate group other than a nucleic acid. The *glycoproteins* yield, upon decomposition, protein and carbohydrate derivatives, notably glycosamine,  $\text{CH}_2\text{OH}-(\text{CHOH})_3\text{CH}(\text{NH}_2)\text{CHO}$ , and galactosamine,  $\text{OHCH}_2-(\text{CHOH})_3\text{CH}(\text{NH}_2)\text{CHO}$ . The principal glycoproteins are *mucoïds*, *mucins*, and *chondroproteins*. By the term *mucoïd* we may in general designate those glycoproteins which occur in tissues, such as *tendomucoïd* from tendinous

<sup>1</sup> Up to this time the yield of 41.32 per cent obtained by Kleinschmitt from hordein was the maximum yield.

issue and *osseomuroid* from bone. The elementary composition of these typical mucoids is as follows:

	N.	S.	C.	H.	O.
Tendomuroid <sup>1</sup> . . . . .	11.75	2.33	48.76	6.53	30.60
Osseomuroid <sup>2</sup> . . . . .	12.22	2.32	47.43	6.03	31.40

The term *mucins* may be said in general to include those forms of glycoproteins which occur in the secretions and fluids of the body. *Seromuroid*<sup>3</sup> is, however, the term applied to the glycoprotein of blood serum. Chondroproteins are so named because *chondromuroid*, the principal member of the group, is derived from cartilage (chondrigen). *Amyloid*,<sup>4</sup> which appears pathologically in the spleen, liver, and kidneys, is also a chondroprotein.

The *nucleoproteins* occur principally in animal and vegetable cells, and following the destruction of these cells they are found in the fluids of the body. These proteins are discharged into the tissue fluids by the activity or disintegration of cells. Combined with the simple protein in the nucleoprotein molecule we find *nucleic acid*, a body which contains phosphorus and which yields *purine bases* and *pyrimidine bases* (*thymine*, *cytosine*, and *uracil*) upon decomposition. The so-called *nucleins* are formed in the gastric digestion of nucleoproteins.

**Wheeler-Johnson Reaction for Uracil and Cytosine.**—To about 5 c.c. of the solution under examination add bromine water until the color is permanent.<sup>5</sup> In case the solution contains only small quantities of cytosine or uracil, it is advisable to remove the excess of bromine by passing a stream of air through the solution. Now add an excess of an aqueous solution of barium hydroxide and note the appearance of a purple color.

Very dilute solutions do not give the test. Under these conditions the solution should be evaporated to dryness, the residue dissolved in a little bromine water and the excess of bromine removed. Then upon adding an excess of barium hydroxide a decided bluish-pink or lavender color will appear in the presence of as small an amount as 0.001 gram of uracil.

In testing solutions for cytosine, it is preferable to warm or boil the solution with bromine water, and after cooling the solution to apply the test as suggested above, being careful to have a slight excess of bromine present before adding barium hydroxide.

<sup>1</sup> Chittenden and Gies: *Jour. Exp. Med.*, 1, 186, 1896.

<sup>2</sup> Hawk and Gies: *Amer. Jour. Physiol.*, 5, 387, 1901.

<sup>3</sup> Bywaters: *Biochemische Zeitschrift*, 15, 322, 1909.

<sup>4</sup> Not to be confused with the substance *amyloid* which may be formed from cellulose (see page 54).

<sup>5</sup> Avoid the addition of a large excess of bromine inasmuch as this will interfere with the test.

The *phosphoproteins* are called *nucleoalbumins* in many classifications and are grouped among the simple proteins. They are considered to be "compounds of the protein molecule and some, as yet undefined, phosphorus-containing substances other than a nucleic acid or lecithin." The percentage of phosphorus in phosphoproteins is very similar to that in nucleoproteins but they differ from this latter class of proteins in that they do not yield any purine bases upon hydrolytic cleavage. Two of the common phosphoproteins are the *caseinogen* of milk and the *ovovitellin* of the egg-yolk.

The *hæmoglobins* (chromoproteins) are compounds of the protein molecule with hæmatin or some similar substance. The principal member of the group is the hæmoglobin of the blood. Upon hydrolytic cleavage this hæmoglobin yields a protein termed *globin* and a coloring matter termed *hæmochromogen*. The latter substance contains *iron* and upon coming in contact with oxygen is oxidized to form *hæmatin*. *Hæmocyanin*, another member of the class of hæmoglobins, occurs in the blood of certain invertebrates, notably cephalopods, gasteropods, and crustacea. Hæmocyanin generally contains either *copper*, *manganese*, or *zinc* in place of the *iron* of the hæmoglobin molecule.

The *lecithoproteins* include such substances as *lecithans* and *phosphatides* which consist of a protein molecule joined to lecithin. They have been comparatively little studied until recently, and in much of the older research they were undoubtedly considered as lecithins.

For experiments on conjugated proteins see pages 63, 162, 247, 251, 271, and 308.

## DERIVED PROTEINS.

These substances are derivatives which are formed through hydrolytic changes of the original protein molecule. They may be divided into two groups, the *primary* protein derivatives and the *secondary* protein derivatives. The term secondary derivatives is made use of in this connection since the formation of the primary derivatives generally precedes the formation of these secondary derivatives. These derived proteins are obtained from native simple proteins by hydrolyses of various kinds, *e. g.*, through the action of acids, alkalis, heat, or enzymes. The particular class of derived protein desired regulates the method of treatment to which the native protein is subjected.

### Primary Protein Derivatives.

The primary protein derivatives are "apparently formed through hydrolytic changes which involve only slight alterations of the protein



molecule." This class includes *proteans*, *metaproteins*, and *coagulated proteins*.

### PROTEANS.

Proteans are those insoluble protein substances which are produced from proteins originally soluble through the incipient action of water, enzymes, or very dilute acids. It is well known that globulins become insoluble upon repeated reprecipitation and it may possibly be found that the greater number of the proteans are transformed globulins. Osborne, however, believes that nearly all proteins may give rise to proteans. This investigator who has so very thoroughly investigated many of the vegetable proteins claims that the hydrogen ion is the active agent in the transformation. The protein produced from the transformation of *edestin* is called *edestan*, that produced from *myosin* is called *myosan*, etc. The name protean was first given to this class of proteins by Osborne in 1900 in connection with his studies of edestin.

### EXPERIMENTS ON PROTEANS.

**Preparation and Study of Edestan.**—Prepare edestin according to the directions given on page 109. Bring the edestin into solution in 0.2 per cent hydrochloric acid and permit the acid solution to stand for about one-half hour.<sup>1</sup> Neutralize, with a 0.5 per cent solution of sodium carbonate, filter off the precipitate of edestan and make the following tests:

1. **Solubility.**—Try the solubility in the ordinary solvents (see page 27). Note the altered solubility of the *edestan* as compared with that of edestin (see page 110).

2. **Millon's Reaction.**

3. **Coagulation Test.**—Place a small amount of the protean in a test-tube, add a little water and boil. Now add dilute hydrochloric acid and note that the protein no longer dissolves. It has been coagulated.

4. **Tests on Edestan Solution.**—Dissolve the remainder of the edestan precipitate in 0.2 per cent hydrochloric acid and make the following tests:

(a) *Biuret Test.*

(b) *Influence of Protein Precipitants.*—Try a few protein precipitants such as *picric acid* and *mercuric chloride*.

### METAPROTEINS.

The metaproteins are formed from the native simple proteins through an action similar to that by which proteans are formed. In the case of

<sup>1</sup> The edestan solution preserved from experiment (5), page 111, may be used.

the *metaproteins*, however, the changes in the original protein molecule are more profound. These derived proteins are characterized by being soluble in very weak acids and alkalis, but *insoluble in neutral fluids*. The metaproteins have generally been termed *albuminates*, but inasmuch as the termination *ate* signifies a *salt* it has always been somewhat of a misnomer.

Two of the principal metaproteins are the *acid metaprotein* or so-called acid albuminate and the *alkali metaprotein* or so-called alkali albuminate. They differ from the native simple proteins principally in being insoluble in sodium chloride solution and in not being coagulated *except when suspended in neutral fluids*. Both forms of metaprotein are precipitated upon the approximate neutralization of their solutions. They are precipitated by saturating their solutions with ammonium sulphate, and by sodium chloride, also, provided they are dissolved in an acid solution. Acid metaprotein contains a higher percentage of nitrogen and sulphur than the alkali metaprotein from the same source, since some of the nitrogen and sulphur of the original protein is liberated in the formation of the latter. Because of this fact, it is impossible to transform an alkali metaprotein into an acid metaprotein, while it is possible to reverse the process and transform the acid metaprotein into the alkali modification.

#### EXPERIMENTS ON METAPROTEINS.

##### ACID METAPROTEIN (ACID ALBUMINATE).

**Preparation and Study.**—Take 25 grams of hashed lean beef washed free from the major portion of blood and inorganic matter, and place it in a medium-sized beaker with 100 c.c. of 0.2 per cent HCl. Place it on a boiling water-bath for one-half hour, filter, *cool*, and divide the filtrate into two parts. Neutralize the *first part* with *dilute* KOH solution, filter off the precipitate of *acid metaprotein* and make the following tests:

- (1) *Solubility*.—Solubility in the ordinary solvents (see page 27).
- (2) *Millon's Reaction*.

(3) *Coagulation Test*.—Suspend a little of the metaprotein in water (neutral solution) and heat to boiling for a few moments. Now add 1–2 drops of KOH solution to the water and see if the metaprotein is still soluble in dilute alkali. What is the result and why?

- (4) *Test for Loosely Combined Sulphur* (see page 108).

Subject the *second part* of the original solution to the following tests:

(1) *Coagulation Test*.—Heat some of the solution to boiling in a test-tube. Does it coagulate?

- (2) *Biuret Test*.

(3) *Influence of Protein Precipitants.*—Try a few protein precipitants such as *picric acid* and *mercuric chloride*. How do the results obtained compare with those from the experiments on egg albumin? (See page 102.)

#### ALKALI METAPROTEIN (ALKALI ALBUMINATE).

**Preparation and Study.**—Carefully separate the white from the yolk of a hen's egg and place the former in an evaporating dish. Add concentrated potassium hydroxide solution, *drop by drop*, stirring continuously. The mass gradually thickens and finally assumes the consistency of jelly. This is *solid alkali metaprotein* or "Lieberkühn's jelly." Do not add an excess of potassium hydroxide or the jelly will dissolve. Cut it into small pieces, place a cloth or wire gauze over the dish, and by means of running water wash the pieces free from adherent alkali. Now add a small amount of water, which forms a weak alkaline solution with the alkali within the pieces, and dissolve the jelly by gentle heat. *Cool* the solution and divide it into two parts. Proceed as follows with the *first part*: Neutralize with *dilute* hydrochloric acid, noting the odor of the liberated hydrogen sulphide as the alkali metaprotein precipitates. Filter off the precipitate and test as for acid metaprotein, page 116, noting particularly the sulphur test. How does this test compare with that given by the acid metaprotein? Make tests on the *second part* of the solution the same as for acid metaprotein, page 116.

#### Coagulated Proteins.

These derived proteins are produced from unaltered protein materials by heat, by long standing under alcohol, or by the continuous movement of their solutions such as that produced by rapid stirring or shaking. In particular instances, such as the formation of fibrin from fibrinogen (see page 195), the coagulation may be produced by enzyme action. Ordinary soluble proteins after having been transformed into the coagulated modification are no longer soluble in the ordinary solvents. Upon being heated in the presence of strong acids or alkalis, coagulated proteins are converted into metaproteins.

Many proteins coagulate at an approximately fixed temperature under definite conditions (see pp. 106 and 254). This characteristic may be applied to separate different coagulable proteins from the same solution by fractional coagulation. The coagulation temperature frequently may serve in a measure to identify proteins in a manner similar to the melting-point or boiling-point of many other organic substances. The separation of proteins by fractional coagulation is thus analogous to the separation of volatile substances by means of *fractional distillation*. This method of

separating proteins is not a satisfactory one, however, inasmuch as proteins in solution have different effects upon one another and also because of the fact that the nature of the solvent causes a variation in the temperature at which a given protein coagulates. The nature of the process involved in the coagulation of proteins by heat is not well understood, but it is probable that in addition to the altered arrangement of the component atoms in the molecule, there is a mild hydrolysis which is accompanied by the liberation of minute amounts of hydrogen, nitrogen, and sulphur. The presence of a neutral salt or a trace of a mineral acid may facilitate the coagulation of a protein solution (see page 106), whereas any appreciable amount of acid or alkali will retard or entirely prevent such coagulation.

It has recently been shown that the coagulation of proteins by heat proceeds in two stages,<sup>1</sup> first, a reaction between the protein and the hot water (denaturation) and second, an agglutination or separation of the altered protein in particulate form. The concentration of acid, or hydrogen ion, in the solution influences the coagulation of proteins, such that the original protein is acted upon less readily by hot water alone than in the presence of acid. The formation of the coagulum is accompanied by the disappearance of the free acid from the solution, indicating the formation of a protein salt. A disturbance of the equilibrium between the hydrolyzed and unhydrolyzed portions of the protein salt, due to the greater rapidity with which the unhydrolyzed portion is precipitated, results in the gradual removal of both protein and acid from the solution. This has been offered as an explanation of the decreasing acidity.

According to Chick and Martin, the addition of neutral salts to the acid solution of the salt-free protein to be coagulated results in a *decreased rate* of coagulation. This is due in part to the decrease in the concentration of the free acid, which results from the disturbance of the equilibrium between the protein and acid and also in part to the direct influence which the salts exert upon the protein. The presence of neutral salts may under certain circumstances facilitate the coagulation of proteins by heat.

The temperature at which egg white is coagulated causes a difference in the appearance of the coagulum.<sup>2</sup> Coagulated egg white which has been immersed in water at a *low* temperature and then gradually heated to the coagulating temperature is more translucent and has a bluish color, whereas, egg white which has been immersed in water heated to a temperature *above* the coagulating temperature is creamy-white in color. The varying digestibility, as the result of the different methods of heating, has been discussed in the chapter on Enzymes.

<sup>1</sup> Chick and Martin: *Journal of Physiology*, 43, 1, 1911.

<sup>2</sup> Frank: *Journal of Biological Chemistry*, 9, 403, 1911

## EXPERIMENTS ON COAGULATED PROTEIN.

Ordinary coagulated egg-white may be used in the following tests:

1. **Solubility.**—Try the solubility of *small* pieces of the coagulated protein in each of the ordinary solvents (see page 27).

2. **Millon's Reaction.**

3. **Xanthoproteic Reaction.**—*Partly* dissolve a medium-sized piece of the protein in concentrated nitric acid. *Cool* the solution and add an excess of ammonium hydroxide. Both the protein solution and the undissolved protein will be colored orange.

4. **Biuret Test.**—*Partly* dissolve a medium-sized piece of the protein in concentrated potassium hydroxide solution. If the proper dilution of copper sulphate solution is now added the white coagulated protein, as well as the protein solution, will assume the characteristic purplish-violet color.

5. **Hopkin's-Cole Reaction.**—Conduct this test according to the modification given on page 98.

## Secondary Protein Derivatives.

These derivatives result from a more profound cleavage of the protein molecule than that which occurs in the formation of the primary derivatives. The class includes *proteoses*, *peptones*, and *peptides*.

## PROTEOSES AND PEPTONES.

Proteoses are intermediate products in the digestion of proteins by proteolytic enzymes, as well as in the decomposition of proteins by hydrolysis and the putrefaction of proteins through the action of bacteria. Proteoses are called *albumoses* by some writers, but it seems more logical to reserve the term *albumose* for the proteose of albumin.

Peptones are formed after the proteoses and it has been customary to consider them as the last product of the processes before mentioned which still possess true protein characteristics. In other words it has been considered that the protein nature of the end-products of the cleavage of the protein molecule ceased with the peptones, and that the simpler bodies formed from peptones were substances of a different nature (see page 70). However, as the end-products have been more carefully studied, it has been found to be no easy matter to designate the exact character of a peptone or to indicate the exact point at which the *peptone* characteristic ends and the *peptide* characteristic begins. The situation regarding the proteoses, peptones and peptides, is at present a most unsatisfactory one because of the unsettled state of our knowledge regarding them. The exact differences between certain members of the peptone

and peptide groups remain to be more accurately established. It has been quite well established that the peptones are peptides or mixtures of peptides but the term peptide is used at present to designate only those possessing a definite structure.

There are several proteoses (protoproteose, heteroproteose and deutero-proteose), and at least two peptones (amphopeptone and antipeptone), which result from proteolysis. The differentiation of the various proteoses and peptones at present in use is rather unsatisfactory. These compounds are classified according to their varying solubilities, especially in ammonium sulphate solutions of different strengths. The exact differences in composition between the various members of the group remain to be more accurately established. Because of the difficulty attending the separation of these bodies, pure proteose and peptone are not easy to procure. The so-called peptones sold commercially contain a large amount of proteose. As a class the proteoses and peptones are very soluble, diffusible bodies which are non-coagulable by heat. *Peptones differ from proteoses in being more diffusible, non-precipitable by  $(\text{NH}_4)_2\text{SO}_4$ , and by their failure to give any reaction with potassium ferrocyanide and acetic acid, potassium-mercuric iodide and HCl, picric acid, and trichloroacetic acid.* The so-called *primary proteoses* are precipitated by  $\text{HNO}_3$  and are the only members of the proteose-peptone group which are so precipitated.

Some of the more general characteristics of the proteose-peptone group may be noted by making the following simple tests on a proteose-peptone powder:

- (1) *Solubility*.—Solubility in the ordinary solvents (see page 27).
- (2) *Millon's Reaction*.

Dissolve a little of the powder in water and test the solution as follows:

(1) *Precipitation by Picric Acid*.—To 5 c.c. of proteose-peptone solution in a test-tube add picric acid until a permanent precipitate forms. The precipitate disappears on heating and returns on cooling.

(2) *Precipitation by a Mineral Acid*.—Try the precipitation by nitric acid.

(3) *Coagulation Test*.—Heat a little proteose-peptone solution to boiling. Does it coagulate like the other simple proteins studied?

#### SEPARATION OF PROTEOSES AND PEPTONES.<sup>1</sup>

Place 50 c.c. of proteose-peptone solution in an evaporating dish or casserole, and *half-saturate* it with ammonium sulphate solution, which

<sup>1</sup> The separation of proteoses and peptones by means of fractional precipitation with ammonium sulphate does not possess the significance it was once supposed to possess inasmuch as the boundary between these substances and *peptides* is not well defined (see p. 119).

may be accomplished by adding an equal volume of *saturated* ammonium sulphate solution. At this point note the appearance of a precipitate of the *primary proteoses* (protoproteose and hetero-proteose). Now heat the half-saturated solution and its suspended precipitate to boiling and *saturate* the solution with *solid* ammonium sulphate. At full saturation the *secondary proteoses* (deuteroproteoses) are precipitated. The peptones remain in solution.

Proceed as follows with the precipitate of proteoses: Collect the sticky precipitate on a rubber-tipped stirring rod or remove it by means of a watch glass to a small evaporating dish and dissolve it in a little water. To remove the ammonium sulphate, which adhered to the precipitate and is now in solution, add barium carbonate, boil, and filter off the precipitate of barium sulphate. Concentrate the proteose solution to a small volume<sup>1</sup> and make the following tests:

(1) *Biuret Test.*

(2) *Precipitation by Nitric Acid.*—What would a precipitate at this point indicate?

(3) *Precipitation by Trichloroacetic Acid.*—This precipitate dissolves on heating and returns on cooling.

(4) *Precipitation by Picric Acid.*—This precipitate also disappears on heating and returns on cooling.

(5) *Precipitation by Potassio-mercuric Iodide and Hydrochloric Acid.*

(6) *Coagulation Test.*—Boil a little in a test-tube. Does it coagulate?

(7) *Acetic Acid and Potassium Ferrocyanide Test.*

The solution containing the peptones should be cooled and filtered, and the ammonium sulphate in solution removed by boiling with barium carbonate as described above. After filtering off the barium sulphate precipitate, concentrate the peptone filtrate to a small volume and repeat the test as given under the proteose solution, above. In the biuret test the solution should be made very strongly alkaline with *solid* potassium hydroxide.

#### PEPTIDES.

The peptides are “definitely characterized combinations of two or more amino acids, the carboxyl (COOH) group of one being united with the amino (NH<sub>2</sub>) group of the other with the elimination of a molecule of water.” These peptides are more fully discussed on pages 71 and 119.

<sup>1</sup> If the proteoses are desired in powder form, this concentrated proteose solution may now be precipitated by alcohol, and this precipitate, after being washed with absolute alcohol and with ether, may be dried and powdered.

## REVIEW OF PROTEINS.

In order to facilitate the student's review of the proteins, the preparation of a chart similar to the model given is recommended. The signs + and - may be conveniently used to indicate positive and negative reactions.

MODEL CHART FOR REVIEW PURPOSES.

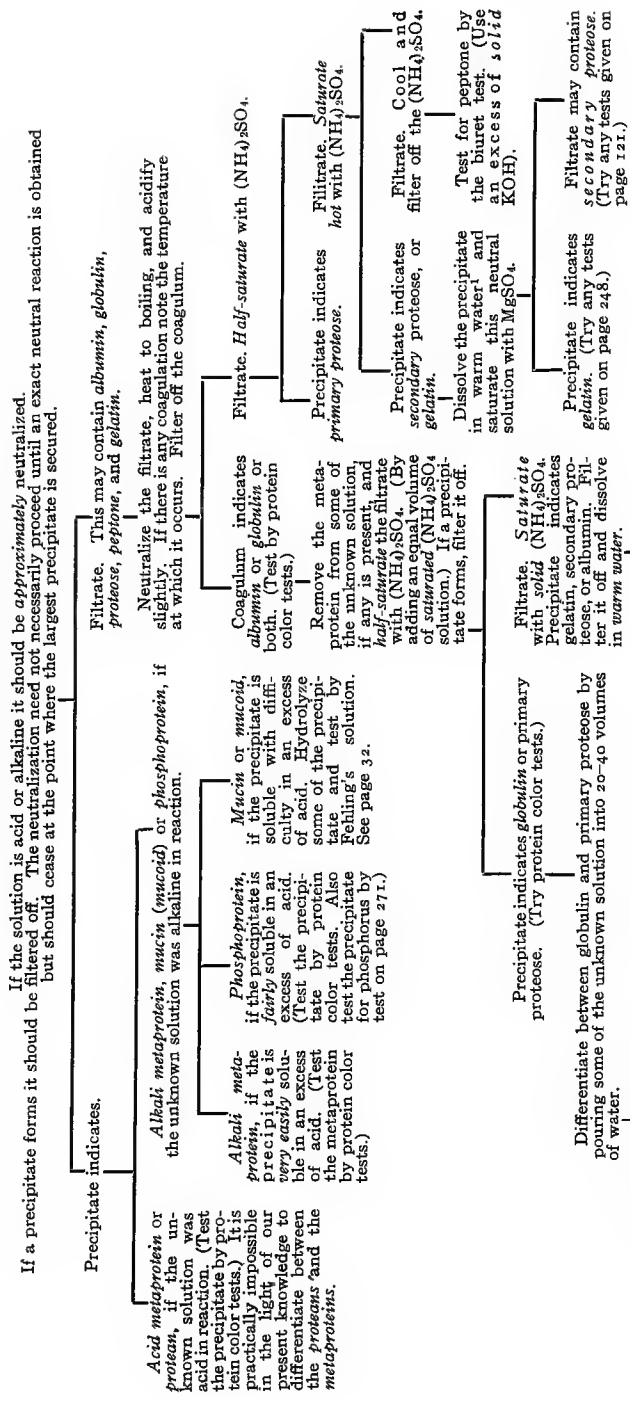
Protein.	Solubility.						Protein Color Test.	Precipitation Tests.						Salting-out Tests.		Diffusion.	Coagulation by Heat.
	Water.	10 % NaCl.	0.2 % HCl.	0.5 % Na <sub>2</sub> CO <sub>3</sub> .	Conc. HCl.	Conc. KOH.		Mineral Acid (HNO <sub>3</sub> ).	Metallic Salt (HgCl <sub>2</sub> ).	Alcohol.	Pot. Ferrocyanide + Acetic Acid.	Potassic-mercuric Iodide + HCl.	Picric Acid.	Trichloroacetic Acid.	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .		
Albumin.																	
Globulin.																	
Protean.																	
Acid metaprotein.																	
Alkali metaprotein.																	
Proteose.																	
Peptone.																	
Coagulated protein.																	

## "UNKNOWN" MIXTURES AND SOLUTIONS OF PROTEINS.

At this point the student's knowledge of the characteristics of the various proteins studied will be tested by requiring him to examine several "unknown" protein mixtures or solutions and make full report upon the same. The scheme given on page 123 may be used in this examination.



SCHEME FOR THE DETECTION OF PROTEINS.



<sup>1</sup> We may also differentiate between gelatin and protease by means of the Hopkins-Cole reaction (see page 98). A positive reaction here would indicate protease and a negative reaction would indicate gelatin.

## CHAPTER VI.

### GASTRIC DIGESTION.

Gastric digestion takes place in the stomach and is promoted by the gastric juice, which is secreted by the glands of the stomach mucosa. These glands are of two kinds, fundus glands and pyloric glands which are situated, as their names imply, in the regions of the fundus and pylorus. The principal foods acted upon in gastric digestion are the proteins which are so changed by its processes as to become better prepared for further digestion in the intestine and for their final absorption.

From reliable experiments made upon lower animals it is evident that the gastric juice is secreted as the result of stimuli of two forms, *i. e.*, *psychical* stimuli and *chemical* stimuli. The psychical form of stimuli may be produced by the sight, thought, or taste of food, and the chemical stimuli may be produced by certain substances, such as water, milk, the extractives of meat, etc., when coming in contact with the stomach mucosa. The stimulatory power of water has been very strikingly demonstrated.<sup>1</sup> Experiments have been made which indicate clearly that the outpouring of gastric juice increases in direct proportion to the volume of water which comes into contact with the gastric mucosa.<sup>2</sup> The claim that the drinking of water with meals is harmful because such a procedure causes a dilution of the gastric juice, has no basis in fact. The drinking of water with meals by normal individuals has been found to be accompanied by a more economical utilization of the ingested proteins, fats and carbohydrates. Various other desirable and *no undesirable* features have been demonstrated as accompanying or following such a dietary procedure.<sup>3</sup> No *experimental* evidence has been submitted which can justly be interpreted as showing any harmful influence to accompany or follow the drinking, by normal persons, of large quantities of water at meal time.

<sup>1</sup> Foster and Lambert: *Journ Exper. Med.*, 10, 820, 1908.

<sup>2</sup> Wills and Hawk: *Jour. Biol. Chem.*, 9, xxx, 1911. (Proceedings).

<sup>3</sup> Hawk: *University of Pennsylvania Medical Bulletin*, 18, 1, 1905.

Fowler and Hawk: *Jour. Exper. Med.*, 12, 388, 1910.

Hattrem and Hawk: *Arch. Int. Med.*, 7, 610, 1911.

Mattill and Hawk: *Jour. Am. Chem. Soc.*, 33, pp. 1978, 1999, and 2019, 1911.

Hawk: *Arch. Int. Med.*, 8, 382, 1911.

Hawk: *Proceedings Soc. Exp. Biol. and Med.*, 8, 36, 1910.

Fairhall and Hawk: *Jour. Am. Chem. Soc.*, 34, 546, 1912.

Howe and Hawk: *Jour. Biol. Chem.*, 11, 129, 1912.

The volume of gastric juice secreted during any given period of digestion, varies with the quantity and kind of the food. These conclusions were deduced principally from a series of so-called *delusive feeding* experiments. A dog was prepared with two œsophageal openings and a gastric fistula. When thus prepared and fed foods of various kinds such as meat and bread, the material instead of passing to the stomach, would invariably find its way out of the animal's body at the upper œsophageal opening. Through the medium of the gastric fistula the course of the secretion of gastric juice could be carefully followed. It was found that when the dog ate meat, for example, there was a large secretion of gastric juice notwithstanding no portion of the food eaten had reached the stomach. Further experiments made through the medium of a *cul-de-sac* formed from the stomach wall have given us many valuable conclusions, among others those regarding the influence of the chemical stimuli. The method followed was to feed the animal certain substances and note the secretion of gastric juice in the miniature stomach while the real process of digestion was taking place in the stomach proper.

Normal gastric juice is a thin, light colored fluid which is acid in reaction and has a specific gravity varying between 1.001 and 1.010. It contains only 2-3 per cent of solid matter which is made up principally of hydrochloric acid, sodium chloride, potassium chloride, earthy phosphates, mucin and the enzymes *pepsin*, *gastric rennin*, and *gastric lipase*; the hydrochloric acid and the enzymes are of the greatest importance. The acidity of the gastric juice is due to *free* hydrochloric. It was formerly believed that this acid was secreted by the parietal cells of the fundus as well as by the chief cells of both the fundus and pyloric glands. It has recently been claimed<sup>1</sup> however, that *the parietal cell is the seat of the formation of the hydrochloric acid*. This conclusion is based upon the formation of Prussian blue after the subcutaneous injection of potassium ferrocyanide and ammonium ferric citrate (rabbits and guinea-pigs) and the subsequent (3 to 30 hours) microscopical examination of the gastric mucosa. The acid was shown to be present in the lumina of the gland tubules and in the canaliculi of the parietal cells; traces were also apparently present in the cytoplasm. Still more recently Bensley and Harvey<sup>2</sup> have shown by means of dyes which act as vital stains and as indicators very sensitive to alkali that *the secretion in the parietal cells is slightly alkaline whereas that in the lumen of the gland proper is very nearly neutral*. Therefore, the acid is formed entirely above the level of the gland proper *i. e.* in the foveolæ and on the surface. It is

<sup>1</sup> Fitzgerald: *Proceedings Royal Society (B)*, 83, 56, 1910.

<sup>2</sup> Bensley and Harvey: Unpublished data furnished by Dr. Bensley.

apparent from the work of Fitzgerald, and Bensley and Harvey that the question as to the seat of formation of the hydrochloric acid must be considered as undecided.

Hydrochloric acid is generally present in the gastric juice of man to the extent of 0.2-0.3 per cent. When the amount of hydrochloric acid varies to any considerable degree from these values a condition of hypoacidity or hyperacidity is established. Hydrochloric acid has the power of combining with protein substances taken in the food, thus forming so-called *combined* hydrochloric acid. This combined acid is a less potent germicide than *free* hydrochloric acid and has less power to destroy the amylolytic enzyme *salivary amylase (ptyalin)* of the saliva. This last fact explains to a degree the possibility of the continuance of salivary digestion in the stomach.

The term *combined* hydrochloric acid is really a misnomer. When *free* hydrochloric acid is treated with a protein the latter functions as a base metal and a *salt* is formed. Therefore, instead of having, "*combined hydrochloric acid*" we have a *protein salt of hydrochloric acid*. This salt ionizes differently from the free acid. This fact explains the variation in the gemicidal properties of the two solutions as well as their different action toward enzymes, such, for example, as salivary amylase (see page 66).

The hydrochloric acid of the gastric juice forms a medium in which the pepsin can most satisfactorily digest the protein food, and at the same time it acts as an antiseptic or germicide which prevents putrefactive processes in the stomach. It also possesses the power of inverting cane sugar, this property being due to the hydrogen ion. When the hydrochloric acid of the gastric juice is diminished in quantity (hypoacidity) or absent, as it may be in many cases of functional or organic disease, there is no check to the growth of micro-organisms in the stomach. There are, however, certain of the more resistant spores which even the normal acidity of the gastric juice will not destroy. A condition of hypoacidity may also give rise to fermentation with the formation of comparatively large amounts of such substances as lactic acid and butyric acid.

The question of the origin of the hydrochloric acid of the gastric juice is a problem to whose solution many investigators have given much attention. Many theories have been proposed, among them being Bunge's *mass action theory*, Köppe's *electrolytic dissociation theory*, and the more recent theory based upon the *interaction of sodium chloride and lactic acid*. We cannot go into a discussion of these various theories. Each of them has met with objection and we have, as yet, no generally accepted theory as to the origin of the hydrochloric acid of the gastric

juice. That this hydrochloric acid originates from the chlorides of the blood is apparently a well established fact, but farther than this no positive statement can be made.

The most important of the enzymes of the gastric juice is the proteolytic enzyme *pepsin*. The pepsin does not originate as such in the gastric cells but is formed from its precursor, the *zymogen* or mother-substance pepsinogen, which is produced by the parietal cells of the fundus as well as by the chief cells of the fundus and pyloric glands. Pepsinogen may be differentiated from pepsin from the fact that it is more resistant to alkali.<sup>1</sup> Upon coming in contact with the hydrochloric acid of the secretion this pepsinogen is immediately transformed into pepsin. Pepsin is not active in alkaline or neutral solutions but requires at least a faint acidity before it can exert its power to dissolve and digest proteins. The percentage of hydrochloric acid facilitating the most rapid peptic action varies with the character of the protein acted upon, *e. g.*, 0.08 per cent to 0.1 per cent for the digestion of fibrin and 0.25 per cent for the digestion of coagulated egg-white. While hydrochloric acid is the acid usually employed to promote artificial peptic proteolysis, other acids, organic and inorganic, will serve the same purpose. Acidity of the liquid is necessary to promote the activity of the pepsin, but the acidity need not necessarily be confined to hydrochloric acid.

In common with many other enzymes pepsin acts best at about 38°-40° C. and its digestive power decreases as the temperature is lowered, the enzyme being only slightly active at 0° C. Its power is only temporarily inhibited by the application of such low temperatures, however, and the enzyme regains its full proteolytic power upon raising the temperature to 40° C. As the temperature of a digestive mixture is raised above 40° C. the pepsin gradually loses its activity until at about 80°-100° C. its proteolytic power is permanently destroyed.

Our ideas regarding the nature of the products formed in the course of peptic proteolysis have undergone considerable revision in recent years. The former view that these products included only acid albuminate (acid metaprotein), proteoses and peptones is no longer tenable. From the investigations of numerous observers we have learned that artificial gastric digestion *if permitted to proceed for a sufficiently long period* will yield, in addition to proteoses and peptones, a long list of protein cleavage products which are crystalline in character, including *leucine, tyrosine, alanine, phenylalanine, aspartic acid, glutamic acid, proline, leucinimide, valine, and lysine*. A similar group of substances may result from the action of the enzyme trypsin (see p. 149). The relative amounts of proteoses, peptones, and crystalline substances

<sup>1</sup> Langley: *Jour. of Physiol.*, 3, p. 246.

formed depends to a great extent upon the character of the protein undergoing digestion, *e. g.*, a greater proportion of proteoses results from the digestion of fibrin than from the digestion of coagulated egg-white. We must not be led into the error of thinking that the large number of protein cleavage products just mentioned are formed in the course of normal gastric digestion *within the animal organism*. They are formed only after comparatively long-continued hydrolysis. In pancreatic digestion, however, there are formed even under normal conditions, the large number of cleavage products to which reference has been made. Peptic proteolysis, therefore, within the animal organism differs from tryptic proteolysis (see page 149) in that the former yields larger amounts of proteoses, smaller amounts of peptones and no considerable quantity of crystalline bodies as end-products in the brief period during which proteins are ordinarily subjected to gastric digestion. Prolonged hydrolysis with gastric juice does, however, yield considerable quantities of the non-protein end-products. In cases of cancer of the stomach a peptide-splitting enzyme (erepsin) is present in the stomach contents. This enzyme is believed to be elaborated by the cancer tissue and its identification is of importance in connection with the diagnosis of gastric cancer. The glycyL-tryptophane test<sup>1</sup> is used for this purpose (see p. 15).

Abderhalden and Meyer<sup>2</sup> have very recently shown active pepsin to be present in the contents of all parts of the small intestine. It is suggested that pepsin may be adsorbed in the stomach by such protein substances as pass into the intestine in solid form and that the pepsin thus protected may bring about gastric digestion whenever the reaction of the surrounding intestinal contents is favorable. This fact may be of importance in connection with the profound proteolysis taking place in the intestine. Heretofore, this process was believed to be furthered alone by trypsin and erepsin. The passage of adsorbed pepsin into the intestine may be an efficient aid to the proper digestion of solid proteins which are ingested without sufficient mastication ("bolted")<sup>3</sup> and which consequently, at times, pass into the intestine in rather large pieces (see chapter on Feces).

*Gastric rennin*, the second enzyme of the gastric juice, is what is known as a *milk curdling* or *protein coagulating* enzyme. Rennin acts upon the caseinogen of the milk, splitting it into a proteose-like body and soluble casein. This soluble body, in the presence of calcium salts, combines with calcium, forming *calcium casein* or true *casein* which is insoluble and precipitates. There is some uncertainty re-

<sup>1</sup> Neubauer and Fischer: *Deut. Arch. f. klin. Med.*, 97, 499, 1909.

<sup>2</sup> Abderhalden and Meyer: *Zeit. für physiol. Chem.*, 74, 67, 1911.

<sup>3</sup> Foster and Hawk: *Proceedings of the Eighth International Congress of Applied Chemistry*, New York, September, 1912.

garding the reaction to litmus in which gastric rennin shows the greatest activity. It is, however, said to be active in neutral, alkaline, or acid solution. However, it probably possesses its greatest activity in the presence of a slight acid reaction, as would naturally be expected. It is especially abundant in the gastric mucosa of the calf, and is used to curdle the milk used in cheese-making. Gastric rennin is always present normally in the gastric juice but in certain pathological conditions such as atrophy of the mucosa, chronic catarrh of the stomach, or in carcinoma it may be absent.

The theory that the proteolytic activity and the milk curdling property of the gastric juice reside in a single substance is causing much controversy at the present time. The theory was originally advanced by the Pawlow school.<sup>1</sup> According to Nencki and Sieber<sup>2</sup> the milk curdling and protein hydrolyzing activities reside in definite and distinct side chains of a single mammoth molecule. The view which has rather the strongest support, however, is to the effect that there are two entirely distinct enzymes. Important evidence has been advanced in favor of this view by Hammarsten,<sup>3</sup> Taylor,<sup>4</sup> and Hemmeter.<sup>5</sup> Very recently Burge<sup>6</sup> has reported experiments upon the influence of a direct electric current upon solutions possessing typical rennin and peptic activities. By this means he was able to prepare a solution possessing strong rennin activity but entirely void of peptic activity. This furnishes strong evidence against the identity of the two enzymes but does not necessarily deny the accuracy of the side-chain theory.

*Gastric lipase*, the third enzyme of the gastric juice, is a fat-splitting enzyme. It possesses but slight activity when the gastric juice is of normal acidity, but evinces its action principally at such times as a gastric juice of low acidity is secreted either from physiological or pathological cause. The digestion of fat in the stomach is, however, at most, of but slight importance as compared with the digestion of fat in the intestine through the action of the lipase of the pancreatic juice (see page 151).

## PREPARATION OF AN ARTIFICIAL GASTRIC JUICE.

Dissect the mucous membrane of a pig's stomach from the muscular portion and discard the latter. Divide the mucous membrane into two parts ( $\frac{4}{5}$  and  $\frac{1}{5}$ ). Cut up the larger portion, place it in

<sup>1</sup> Pawlow and Parastschuk: *Zeitschrift für Physiologische Chemie*, 42, 415, 1904.

<sup>2</sup> Nencki and Sieber: *Zeitschrift für Physiologische Chemie*, 23, 291, 1901.

<sup>3</sup> Hammarsten: *Zeitschrift für Physiologische Chemie*, 56, 18, 1908.

<sup>4</sup> Taylor: *Journal of Biological Chemistry*, 5, 399, 1909.

<sup>5</sup> Hemmeter: *Berliner klinische Wochenschrift*, Ewald Festnummer, 44, 1905.

<sup>6</sup> Burge: *American Journal of Physiology*, 29, 1912.

a large-sized beaker with 0.4 per cent hydrochloric acid and keep at 38°-40° C. for at least 24 hours. Filter off the residue, consisting principally of nuclein and anti-albumid, and use the filtrate as an artificial gastric juice. This filtrate contains pepsin, rennin, and the products of the digestion of the stomach tissue, *i. e.*, acid metaprotein (acid albuminate), proteoses, peptones, etc.

#### PREPARATION OF A GLYCEROL EXTRACT OF PIG'S STOMACH.

Take the one-fifth portion of the mucous membrane of the pig's stomach not used in the preparation of the artificial gastric juice, cut it up very finely, place it in a small-sized beaker and cover the membrane with glycerol. Stir frequently and allow to stand at room temperature for at least 24 hours. The glycerol will extract the *pepsinogen*. Separate, with a pipette or by other means, the glycerol from the pieces of mucous membrane and use the glycerol extract as required in the later experiments.

#### PRODUCTS OF GASTRIC DIGESTION.

Into the artificial gastric juice, prepared as above described, place the protein material (fibrin, coagulated egg-white, or lean beef) provided for you by the instructor, add 0.4 per cent hydrochloric acid as suggested by the instructor and keep the digestion mixture at 40° C. for 2 to 3 days. Stir frequently and keep *free* hydrochloric acid present in the solution (for tests for free hydrochloric acid see p. 131).

The original protein has been digested and the solution now contains the products of peptic proteolysis, *i. e.*, acid metaprotein (acid albuminate), proteoses, peptones, etc. The insoluble residue may include nuclein and anti-albumid. Filter the digestive mixture and after testing for *free* hydrochloric acid neutralize the filtrate with potassium hydroxide solution. If any of the acid metaprotein (acid albuminate) is still untransformed into proteoses it will precipitate upon neutralization. If any precipitate forms heat the mixture to boiling, and filter. If no precipitate forms proceed without filtering.

We now have a solution containing a mixture consisting principally of proteoses and peptones. Separate and identify the proteoses and peptones according to the directions given on pages 120 and 121.

#### Tests for Free and Combined HCl.

These tests are made with a class of reagents known as *indicators*, so-called because they show changes of color according to the degree of acidity (or alkalinity) of the solution. They behave as though they were weak acids or bases whose ions and unionized molecules have



different colors. Modern theories of color in organic compounds however class them as tautomeric substances.

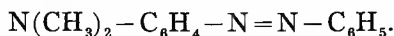
A neutral solution is one in which there are equal numbers of hydrogen and hydroxyl ions. An acid solution has a preponderance of hydrogen ion and an alkaline solution an excess of hydroxyl ion. All indicators do not show changes of color at the true neutral point, but at some fixed degree of acidity (or alkalinity), *i. e.*, at a definite hydrogen or hydroxyl ion concentration. Those indicators which change color at the approximate true neutral point are litmus and rosolic acid, while phenolphthalein changes color in a slightly alkaline solution. Congo red, sodium alizarin sulphonate and tropæolin OO are examples of indicators which change color in an acid solution.

Organic acids are not sufficiently strong, *i. e.*, do not produce enough hydrogen ion, to cause color changes with the last-mentioned class of indicators; litmus, rosolic acid, and phenolphthalein however indicate the hydrogen ion concentration of organic acids or their solutions. Even very dilute solutions of mineral acids are sufficiently acid to produce color changes with congo red, etc. Phenolphthalein, which changes color in a weakly alkaline solution, is used to indicate the presence of acid combined with weakly alkaline substances (as protein) as well as the other types of acid and, hence, is used to indicate the total acidity. The differentiation between the various forms of acidity depends upon the above facts.

The hydrogen ion concentrations at which some common indicators show the most characteristic change of color are given below. Concentrations are expressed in approximate moles of hydrogen ion per liter.

Indicator.	Hydrogen ion concentration.	True nature of solution when the color changes.
Rosolic acid.....	$1 \times 10^{-7}$ .....	Neutral.
Litmus.....	Between $1 \times 10^{-6}$ and $1 \times 10^{-7}$ ..	Neutral.
Tropæolin OO.....	$1 \times 10^{-2}$ .....	Acid.
Dimethyl-amino-azobenzene ..	Between $1 \times 10^{-3}$ and $1 \times 10^{-4}$ ..	Acid.
Sodium alizarin sulphonate....	Between $1 \times 10^{-5}$ and $1 \times 10^{-6}$ ..	Acid.
Congo red.....	Between $1 \times 10^{-5}$ and $1 \times 10^{-6}$ ..	Acid.
Phenolphthalein.....	Between $1 \times 10^{-8}$ and $1 \times 10^{-9}$ ..	Alkaline.

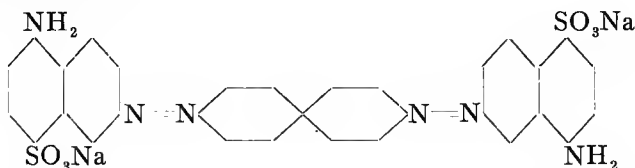
Examine each of the following solutions by means of the tests given below and report the results in a form similar to the chart given on page 133: (1) 0.2 per cent *free* hydrochloric acid. (2) 0.05 per cent *free* hydrochloric acid. (3) 0.01 per cent *free* hydrochloric acid. (4) 0.05 per cent *combined* hydrochloric acid (see p. 126). (5) 1 per cent lactic acid. (6) Equal volumes of 0.2 per cent *free* hydrochloric acid and 1 per cent lactic acid. (7) 1 per cent potassium hydroxide.

1. **Dimethyl-amino-azobenzene (or Töpfer's Reagent),<sup>1</sup>**

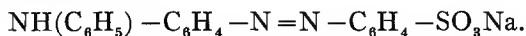
Place 1-2 drops of the reagent in the solution to be tested. Free mineral acid (hydrochloric acid) is indicated by the production of a pinkish-red color. If free acid is absent a yellow color ordinarily results.

2. **Günzberg's Reagent.<sup>2</sup>**—Place 1-2 drops of the reagent in a small porcelain evaporating dish and *carefully* evaporate to dryness over a *low* flame. Insert a glass stirring rod into the mixture to be tested and draw the moist end of the rod through the dried reagent. Warm again gently and note the production of a purplish-red color in the presence of *free* hydrochloric acid.

3. **Boas' Reagent.<sup>3</sup>**—Perform this test in the same manner as 2, above. Free hydrochloric acid is indicated by the production of a rose-red color which becomes less pronounced on cooling.

4. **Congo Red,<sup>4</sup>**

Conduct this test according to the directions given under 1 or 2, above. A blue color indicates free hydrochloric acid, a violet color indicates an organic acid and a brown color indicates combined hydrochloric acid. Congo-red paper, made by immersing ordinary filter paper in the indicator and subsequently drying, may be used in this test.

5. **Tropæolin OO,<sup>5</sup>**

Place 2 drops of the solution to be tested and 1 drop of the indicator in an evaporating dish and evaporate to dryness over a low flame. The formation of a reddish-violet color indicates *free* hydrochloric acid.

This test may also be conducted in the same manner as 2, above.

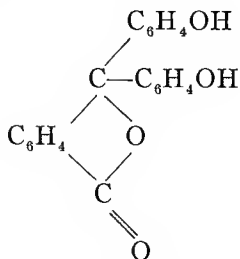
<sup>1</sup> To prepare Töpfer's reagent dissolve 0.5 gram of dimethyl-amino-azobenzene in 100 c.c. of 95 per cent alcohol.

<sup>2</sup> Günzberg's reagent is prepared by dissolving 2 grams of phloroglucinol and 1 gram of vanillin in 100 c.c. of 95 per cent alcohol.

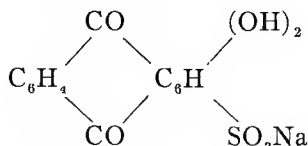
<sup>3</sup> Boas' reagent is prepared by dissolving 5 grams of resorcinol and 3 grams of sucrose in 100 c.c. of 50 per cent alcohol.

<sup>4</sup> This indicator is prepared by dissolving 0.5 gram of congo red in 90 c.c. of water and adding 10 c.c. of 95 per cent alcohol.

<sup>5</sup> Prepared by dissolving 0.05 gram of tropæolin OO in 100 c.c. of 50 per cent alcohol.

6. Phenolphthalein,<sup>1</sup>

Add the indicator directly to the solution, or apply the test according to the directions given under 2 on page 134. This indicator serves to denote the *total acidity* since it is acted upon by free mineral acids, combined acids, organic acids, and acid salts. A red color indicates the presence of an alkali and the indicator is colorless in the presence of a neutral or acid reaction. This indicator is unsatisfactory in the presence of ammonia.

7. Sodium Alizarin Sulphonate,<sup>2</sup>

This indicator may be used directly in the solution to be tested, or the test may be applied as 2, page 134. It serves to indicate all acid reactions except those due to *combined* acids. A reddish-violet color indicates an alkaline reaction, while a yellow color indicates an acid reaction due to a free mineral acid, an organic acid, or an acid salt.

Report the results of your tests tabulated in the form given below:

Name of Indicator.	Solutions Examined.						
	0.2% HCl.	0.05% HCl.	0.01% HCl.	0.05% Combined HCl.	1% Lactic Acid.	Equal Vols. 0.2% HCl and 1% Lactic Acid.	1% KOH.
Töpfer's Reagent.							
Günzberg's Reagent.							
Boas' Reagent.							
Congo Red.							
Tropæolin OO.							
Phenolphthalein.							
Alizarin.							

<sup>1</sup> This indicator is prepared by dissolving 1 gram of phenolphthalein in 100 c.c. of 95 per cent alcohol.

<sup>2</sup> Prepare this indicator by dissolving 1 gram of sodium alizarin sulphonate in 100 c.c. of water.

## GENERAL EXPERIMENTS ON GASTRIC DIGESTION.

1. **Conditions Essential for the Action of Pepsin.**—Prepare four test-tubes as follows:

- (a) Five c.c. of pepsin solution.
- (b) Five c.c. of 0.4 per cent hydrochloric acid.
- (c) Five c.c. of pepsin-hydrochloric acid solution.
- (d) Two or three c.c. of pepsin solution and 2–3 c.c. of 0.5 per cent sodium carbonate solution.

Introduce into each tube a small piece of fibrin and place them in the incubator or water-bath at 40° C. for one-half hour, carefully noting any changes which occur.<sup>1</sup> Now combine the contents of tubes (a) and (b) and see if any further change occurs after standing at 40° C. for 15–20 minutes. Explain the results obtained from these five experiments.

2. **Influence of Different Temperatures.**—In each of four test-tubes place 5 c.c. of pepsin-hydrochloric acid solution. Immerse one tube in cold water from the faucet, keep a second tube at room temperature and place a third in the incubator or water-bath at 40° C. Boil the contents of the fourth tube for a few moments, then cool and also keep it at 40° C. Into each tube introduce a small piece of fibrin and note the progress of digestion. In which tube does the most rapid digestion occur? Explain this.

3. **The Most Favorable Acidity.**—Prepare three tubes as follows:

- (a) Five c.c. of 0.2 per cent pepsin-hydrochloric acid solution.
- (b) Two or three c.c. of 0.2 per cent hydrochloric acid + 1 c.c. of concentrated hydrochloric acid + 5 c.c. of pepsin solution.
- (c) One c.c. of 0.2 per cent pepsin-hydrochloric acid solution + 5 c.c. of water.

Introduce a small piece of fibrin into each tube, keep them at 40° C., and note the progress of digestion. In which degree of acidity does the fibrin digest the most rapidly?

4. **Differentiation Between Pepsin and Pepsinogen.**—Prepare five tubes as follows:

- (a) Few drops of glycerol extract of pepsinogen + 2–3 c.c. of water.
- (b) Few drops of glycerol extract of pepsinogen + 5 c.c. of 0.2 per cent hydrochloric acid.

<sup>1</sup> Digestion of fibrin in a pepsin-hydrochloric acid solution is indicated first by a *swelling* of the protein due to the action of the acid, and later by a *disintegration* and *dissolving* of the fibrin due to the action of the pepsin-hydrochloric acid. If uncertain at any time whether digestion has taken place, the solution under examination may be filtered and the biuret test applied to the filtrate. A positive reaction will signify the presence of acid metaprotein (acid albuminate), proteoses (albumoses), or peptones, the presence of any one of which would indicate that digestion has taken place.

(c) Few drops of glycerol extract of pepsinogen + 5 c.c. of 0.5 per cent sodium carbonate.

(d) Two or three c.c. of pepsin solution + 2-3 c.c. of 1 per cent sodium carbonate.

(e) Few drops of glycerol extract of pepsinogen + 5 c.c. of 1 per cent sodium carbonate.

Add a small piece of fibrin to the contents of each tube, keep the five tubes at 40° C. for one-half hour and observe any changes which may have occurred. To (a) add an equal volume of 0.4 per cent hydrochloric acid, neutralize (c), (d) and (e) with hydrochloric acid and add an equal volume of 0.4 per cent hydrochloric acid. Place these tubes at 40° C. again and note any further changes which may occur. What contrast do we find in the results from the last three tubes? Why is this so?

5. **Comparative Digestive Power of Pepsin with Different Acids.**—Prepare a series of tubes each containing one of the following acids: 0.5 per cent acetic, lactic, oxalic, salicylic, tannic, and butyric, and 0.2 per cent hydrochloric, sulphuric, nitric, arsenious, and *combined* hydrochloric. To each acid add a few drops of the glycerol extract of pig's stomach and a small piece of fibrin. Shake well, place at 40° C., and note the progress of digestion. In which tubes does the most rapid digestion occur?

6. **Influence of Metallic Salts, etc.**—Prepare a series of tubes and into each tube introduce 4 c.c. of pepsin-hydrochloric acid solution and 1/2 c.c. of one of the chemicals listed in Experiment 18 under Salivary Digestion, page 66. Introduce a small piece of fibrin into each of the tubes and keep them at 40° C. for one-half hour. Note the variations in the progress of digestion. Where has the least rapid digestion occurred?

7. **Sahli's Desmoid Reaction.**—This is a method for testing gastric function without using the stomach tube. The underlying principle of the test is the fact that raw catgut may be digested in gastric juice but is entirely indigestible in pancreatic juice. The test is made as follows: A methylene-blue pill is introduced into a small rubber bag and the mouth of the bag subsequently tied with catgut.<sup>1</sup> The small bag is then ingested immediately after the mid-day meal and the urine examined 5, 7, 9 and 18-20 hours later for methylene blue. If methylene

<sup>1</sup> About 0.05 gram of methylene blue is mixed with sufficient *ext. glycyrrhizæ* to form a pill about 3-4 mm. in diameter. The pill is then placed in the center of a square piece of thin rubber dam and a little bag-like receptacle constructed by a twisting movement. The neck of the bag is then closed by wrapping three turns of catgut about it. The most satisfactory catgut to use is *number 00 raw catgut* which has previously been soaked in water until soft. When ready for use the bag should sink instantly when placed in water and be water-tight.

blue is present in appreciable quantity, it will impart to the urine a greenish-blue color. If not present in sufficient amount to impart this color the urine should be boiled with  $\frac{1}{5}$  its volume of glacial acetic acid, whereupon a greenish-blue color results if the chromogen of methylene blue is present. This contingency seldom arises, however, inasmuch as in most cases of uncolored urine it will be found that the rubber bag has passed through the stomach unopened. If the methylene blue is found in the urine inside of 18–20 hours a satisfactory gastric function is indicated.

For Einhorn's bead method for the study of digestive function, see chapter on Feces.

### 8. Testing the Motor and Functional Activities of the Stomach.

—This test is performed the same as Experiment 19 under Salivary Digestion, page 67. If the experiment was carried out under salivary digestion it will not be necessary to repeat it here.

9. **Influence of Bile.**—Prepare five tubes as follows:

(a) Five c.c. of pepsin-hydrochloric acid solution +  $\frac{1}{2}$ –1 c.c. of bile.

(b) Five c.c. of pepsin-hydrochloric acid solution + 1–2 c.c. of bile.

(c) Five c.c. of pepsin-hydrochloric acid solution + 2–3 c.c. of bile.

(d) Five c.c. of pepsin-hydrochloric acid solution + 5 c.c. of bile.

(e) Five c.c. of pepsin-hydrochloric acid solution.

Introduce into each tube a small piece of fibrin. Keep the tubes at 40° C. and note the progress of digestion. Does the bile exert any appreciable influence? How?

10. **Influence of Gastric Rennin on Milk.**—Prepare a series of five tubes as follows:

(a) Five c.c. of fresh milk + 0.2 per cent hydrochloric acid (add slowly until precipitate forms).

(b) Five c.c. of fresh milk + 5 drops of rennin solution.

(c) Five c.c. of fresh milk + 10 drops of 0.5 per cent sodium carbonate solution.

(d) Five c.c. of fresh milk + 10 drops of a saturated solution of ammonium oxalate.

(e) Five c.c. of fresh milk + 5 drops of 0.2 per cent hydrochloric acid. Now to each of the tubes (c), (d), and (e) add 5 drops of rennin solution. Place the whole series of five tubes at 40° C. and after 10–15 minutes note what is occurring in the different tubes. Give a reason for each particular result.

11. **Tests for Lactic Acid.**—(a) *Uffelmann's Reaction.*—To a small

quantity of Uffelmann's reagent<sup>1</sup> in a test-tube add a few drops of a lactic acid solution. The amethyst-blue color of the reagent is displaced by a straw yellow. Other organic acids give a similar reaction. Mineral acids such as hydrochloric acid discharge the blue coloration leaving a colorless solution. In other words, the color of the reagent is weakened in the presence of an *acid* reaction.

(b) *Ferric Chloride Test*.—Place 10 c.c. of *very dilute* ferric chloride in each of five tubes. To the first add 2 c.c. of 0.2 per cent hydrochloric acid, to the second 2 c.c. of 10 per cent alcohol, to the third 2 c.c. of 2 per cent sucrose, to the fourth 2 c.c. of lactic acid and to the fifth 2 c.c. of peptone solution.

It is evident from the results obtained that neither of the tests given above is satisfactory for the detection of lactic acid in the presence of other substances such as we find in the gastric contents.

A satisfactory deduction regarding the presence of lactic acid can only be made after extracting the gastric contents with ether, evaporating the ether extract to dryness, and dissolving the residue in water. This residue will not contain any of the contaminations which interfere with the simple tests as tried above, and therefore if either of the tests is now tried on the dissolved residue of the ether extract we may form an accurate conclusion regarding the presence of lactic acid.

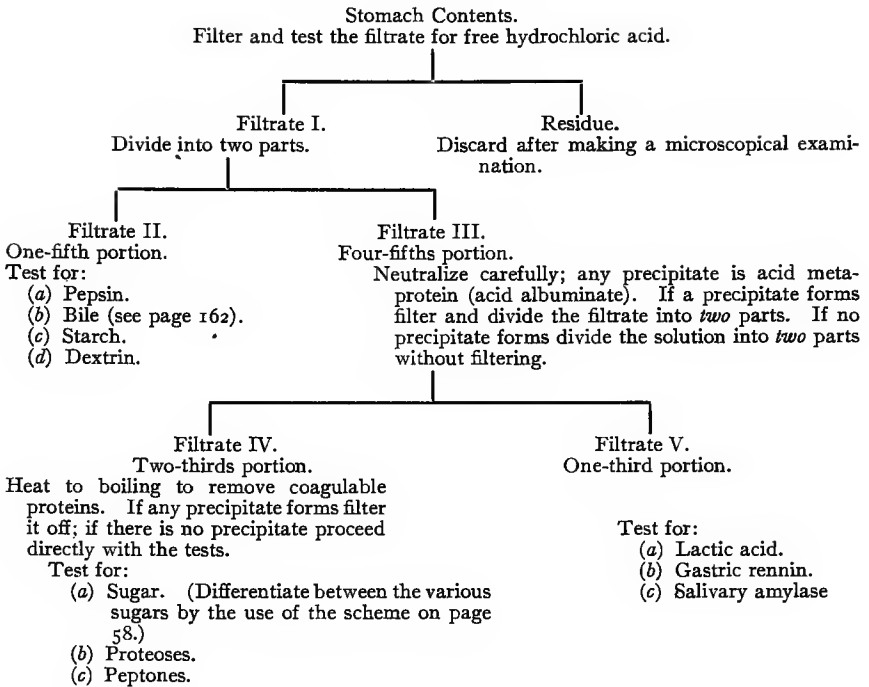
(c) *Hopkins' Thiophene Reaction*.—Place about 5 c.c. of concentrated sulphuric acid in a test-tube and add one drop of a saturated solution of copper sulphate.<sup>2</sup> Introduce a few drops of the solution to be tested, shake the tube well, and immerse it in the boiling water of a beaker-water-bath for one or two minutes. Now remove the tube, cool it under running water, add 2–3 drops of a dilute alcoholic solution<sup>3</sup> of thiophene,  $C_4H_4S$ , from a pipette, replace the tube in the beaker and carefully observe any color change which may occur. Lactic acid is indicated by the appearance of a bright *cherry-red* color which forms rapidly. This color may be made more or less permanent by cooling the tube as soon as the color is produced. Excess of thiophene produces a deep yellow or brown color with sulphuric acid. The test is not wholly specific though the author claims it to be more so than Uffelmann's reaction.

12. **Qualitative Analysis of Stomach Contents**.—Take 100 c.c. of stomach contents and analyze it according to the following scheme:

<sup>1</sup> Uffelmann's reagent is prepared by adding ferric chloride solution to a 1 per cent solution of carbolic acid until an amethyst-blue color is obtained, due to the formation of a ferric salt of carbolic acid.

<sup>2</sup> This is added to catalyze the oxidation which follows.

<sup>3</sup> About 10–20 drops in 100 c.c. of 95 per cent alcohol.





## CHAPTER VII.

### FATS.

Fats occur very widely distributed in the plant and animal kingdoms, and constitute the third general class of food stuffs. In plant organisms they are to be found in the seeds, roots, and fruit while each individual tissue and organ of an animal organism contains more or less of the substance. In the animal organism fats are especially abundant in the bone marrow and adipose tissue. They contain the same elements as the carbohydrates, *i. e.*, carbon, hydrogen, and oxygen, but the oxygen is present in smaller percentage than in the carbohydrates and the hydrogen and oxygen are not present in the proportion to form water.

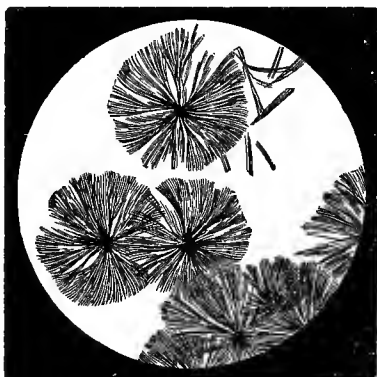
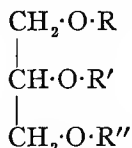


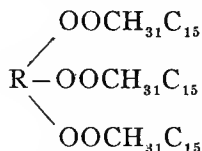
FIG. 36.—BEEF FAT. (*Long.*)

Chemically considered the fats are esters<sup>1</sup> of the tri-atomic alcohol, glycerol, and the mono-basic fatty acids. In the formation of these fats three molecules of water result. This water may arise in either of two ways. First, by the replacement of the H of each of the OH groups of glycerol by a fatty acid radical, giving the following formula in which R, R' and R'' represent fatty acid radicals,



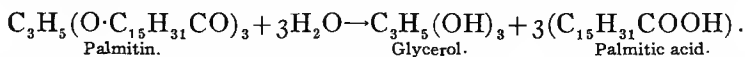
<sup>1</sup> An ester is an oxyacid, one of whose acid hydrogens is replaced by an organic radical

Second, by the replacement of the H's of the carboxyl groups of the three fatty acid molecules by the glycerol radical, thus yielding the following type of formula in which R represents the glycerol radical,



Of these two processes the second is the more logical procedure from the standpoint of the ionic theory. The three fatty acid radicals entering into the structure of a neutral fat may be the radicals of the same fatty acid or they may consist of the radicals of three different fatty acids.

By hydrolysis of a neutral fat, *i. e.*, by the addition to the molecule of those elements which are eliminated in the formation of the fat from glycerol and fatty acid, it may be resolved into its component parts, *i. e.*, glycerol and fatty acid. In the case of palmitin the following would be the reaction:



This process is called *saponification* and may be produced by boiling with alkalis; by the action of steam under pressure; by long-continued contact with air and light; by the action of certain bacteria and by fat-splitting enzymes or lipases, *e. g.*, *pancreatic lipase* (see page 151). The cells forming the walls of the intestines evidently possess the peculiar property of synthesizing the glycerol and fatty acid thus formed so that after absorption these bodies appear in the blood not in their individual form but as neutral fats. This synthesis is similar to that enacted in the absorption of protein material where the peptones are synthesized into albumin in the act of absorption.

The principal animal fats with which we have to deal are *stearin*, *palmitin*, *olein*, and *butyryn*. Such less important forms as laurin and myristin may occur abundantly in plant organisms. The older system of nomenclature for these fats was to apply the prefix "tri" in each case (*e. g.*, *tripalmitin*) since there<sup>where</sup> fatty acid radicals are contained in the neutral fat molecule.

Fats occur ordinarily as mixtures of several individual fats. For example, the fat found in animal tissues is a mixture of olein, palmitin and stearin, the percentage of any one of these fats present depending upon the particular species of animal from whose tissue the fat was derived. Thus the ordinary mutton fat contains more stearin and less olein than the pork fat. Human fat contains from 67 per cent to 85 per

cent of olein and according to Benedict and Osterberg, upon analysis yields 76.08 per cent of carbon and 11.78 per cent of hydrogen. Butter consists in large part of olein and palmitin. Stearin, butyrin, caproin and traces of other fats are also present.

Pure neutral fats are odorless, tasteless, and generally colorless. They are insoluble in the ordinary protein solvents such as water, salt solutions, and dilute acids and alkalis, but are very readily soluble in ether, benzene, chloroform, and *boiling* alcohol. The neutral fats are non-volatile substances possessing a *neutral* reaction. If allowed to remain in contact with the air for a sufficient length of time they become yellow in color, assume an *acid* reaction and are said to be *rancid*. The neutral fats may be crystallized, some of them with great facility. The crystalline forms of some of the more common fats are reproduced in Figs. 36, 37 and 38 on pages 139, 142 and 144. Each individual fat possesses a specific melting- or boiling-point (according to whether the body is solid or fluid in character) and this property of melting or boiling at a definite temperature may be used as a means of differentiation in the same way as the coagulation temperature (see page 117) is used for the differentiation of coagulable proteins. When shaken with water, or a solution of albumin, soap, or acacia, the liquid fats are finely divided and assume a condition known as an *emulsion*. The emulsion with water is transitory, while the emulsions with soap, acacia, or albumin, are permanent.

The fat ingested continues essentially unaltered until it reaches the intestine where it is acted upon by *pancreatic lipase (steapsin)* the fat-splitting enzyme of the pancreatic juice (see page 151), and glycerol and fatty acid are formed from a large portion of the fat. Part of the fatty acid thus formed is dissolved in the bile and absorbed while the remainder unites with the alkalis of the pancreatic juice and forms soluble soaps. These soaps may further act to produce an emulsion of the remaining fat and thus aid in its absorption. That bile is of assistance in the absorption of fat is indicated by the increase of fat in the feces when for any reason bile does not pass into the intestine. That fat is not absorbed unsplit in the form of an emulsion has recently been redemonstrated by Whitehead<sup>1</sup> in a histological study of the absorption in the cat's intestine of fat stained with Sudan III. Whitehead considers that fat was not absorbed unsplit because *no dye was found in the lacteals*. Mendel<sup>2</sup> has pointed out that Sudan III is soluble in fatty acids as well as fats, and therefore its presence in the lacteals furnishes no evidence "for or against the possibility of the absorption of fats prior to their digestion." The failure to find Sudan III in the lacteals may have been due to the

<sup>1</sup> Whitehead: *American Journal of Physiology*, 24, 294, 1909.

<sup>2</sup> Mendel: *Ibid.*, p. 493.

fact that in *postmortem* examinations these vessels are often found collapsed and empty.

The fat distributed throughout the animal body is formed partly from the ingested fat and partly from carbohydrates and the "carbon moiety" of protein material. The formation of *adipocere* and the occurrence of *fatty degeneration* are sometimes given as proofs of the formation of fat from protein. This is questioned by many investigators. Rather more satisfactory and direct proof of the formation of fat from protein material has been obtained by Hofmann in experimentation



FIG. 37.—MUTTON FAT. (Long.)

with *fly-maggots*. The normal content of fat in a number of maggots was determined and later the fat content of others which had developed in blood (84 per cent of the solid matter of blood plasma is protein material) was determined. The fat content was found to have increased 700 to 1100 per cent as a result of the diet of blood proteins. The celebrated experiments of Pettenkofer and Voit, however, have furnished what is, perhaps, the most substantial positive evidence of the formation of fat from protein. These investigators fed dogs large amounts of lean meat, daily, and through subsequent urinary and fecal examinations were enabled to account for *only part of the ingested carbon*, although obtaining a satisfactory nitrogen balance. The discrepancy in the carbon balance was explained upon the theory that the protein of the ingested meat had been split into a *nitrogenous* and a *non-nitrogenous* portion in the organism, and that the non-nitrogenous portion, the so-called "carbon moiety" of the protein, had been subsequently transformed into fat and deposited as such in the tissues of the organism. Some investigators are not inclined to accept these data regarding the formation of fat from protein as conclusive.

Later evidence in favor of the formation of fat from protein has been furnished by the experiments of Weinland. This investigator worked with the larvæ of *Calliphora*,<sup>1</sup> these larvæ being rubbed up in a mortar<sup>2</sup> with Witte's peptone and water to form a homogeneous mixture. After placing these mixtures at 38° C. for 24 hours the fat content was found to have increased, as much as 140 per cent in some instances. The active agency in this transformation of fat is the *larval tissue* since the tissues of both the dead and living larvæ possess the property. Data are given from control tests which show that the action of bacteria in this transformation of protein was excluded.

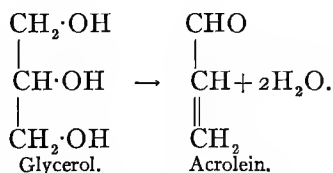
#### EXPERIMENTS ON FATS.

1. **Solubility.**—Test the solubility of olive oil in each of the ordinary solvents (see page 27) and in cold alcohol, hot alcohol, chloroform, ether, and carbon tetrachloride.

2. **Formation of a Transparent Spot on Paper.**—Place a drop of olive oil upon a piece of ordinary writing paper. Note the transparent appearance of the paper at the point of contact with the fat.

3. **Reaction.**—Try the reaction of *fresh* olive oil to litmus, congo red and phenolphthalein. Repeat the test with *rancid* olive oil. What is the reaction of a fresh fat and how does this reaction change upon allowing the fat to stand for some time?

4. **Formation of Acrolein.**—To a little olive oil in a mortar add some dry potassium bisulphate,  $\text{KHSO}_4$ , and rub up thoroughly. Transfer to a *dry* test-tube and cautiously heat. Note the irritating odor of *acrolein*. The glycerol of the fat has been dehydrolyzed and acrylic aldehyde or acrolein has been produced. This is the reaction which takes place:



5. **Emulsification.**—(a) Shake up a drop of *neutral*<sup>3</sup> olive oil with a little water in a test-tube. The fat becomes finely divided, forming an emulsion. This is not a permanent emulsion since the fat separates and rises to the top upon standing.

<sup>1</sup> The ordinary "blow-fly."

<sup>2</sup> Intact larvæ were used in some experiments.

<sup>3</sup> Neutral olive oil may be prepared by shaking ordinary olive oil with a 10 per cent solution of sodium carbonate. This mixture should then be extracted with ether and the ether removed by evaporation. The residue is *neutral* olive oil.

(b) To 5 c.c. of water in a test-tube add 2 or 3 drops of 0.5 per cent  $\text{Na}_2\text{CO}_3$ . Introduce into this faintly alkaline solution a drop of *neutral* olive oil and shake. The emulsion while not permanent is not so transitory as in the case of water free from sodium carbonate.

(c) Repeat (b) using *rancid* olive oil. What sort of an emulsion do you get and why?

(d) Shake a drop of *neutral* olive oil with dilute albumin solution. What is the nature of this emulsion? Examine it under the microscope.

6. **Fat Crystals.**—Dissolve a small piece of lard in ether in a test-tube, add an equal volume of alcohol and allow the alcohol-ether mixture

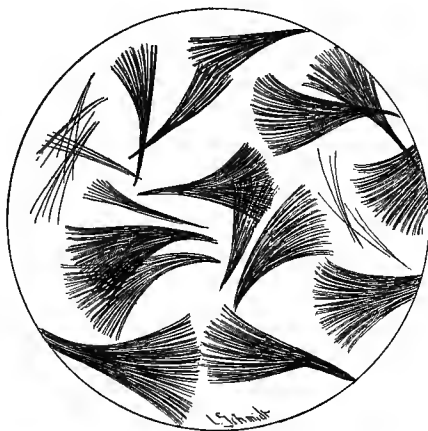


FIG. 38.—PORK FAT.

to evaporate spontaneously. Examine the crystals under the microscope and compare them with those reproduced in Figs. 36, 37 and 38, on pages 39, 142 and 144.

7. **Saponification of Bayberry Tallow.**<sup>1</sup>—Fill a large casserole two-thirds full of water rendered strongly alkaline with *solid* potassium hydroxide (a stick one inch in length). Add about 10 grams of bayberry tallow and boil, keeping the volume constant by adding water as needed. When saponification is complete<sup>2</sup> remove 25 c.c. of the soap solution for use in Experiment 8 and add concentrated hydrochloric acid slowly to the remainder until no further precipitate is produced.<sup>3</sup> Cool the solution and the precipitate of free fatty acid will rise to the surface and form a cake. In this instance the fatty acid is principally *pal-*

<sup>1</sup> Bayberry tallow is derived from the fatty covering of the berries of the *wax myrtle*. It is therefore frequently called "myrtle wax" or "bayberry wax."

<sup>2</sup> Place 2 or 3 drops in a test-tube full of water. If saponification is complete the products will remain in solution and no oil will separate.

<sup>3</sup> Under some conditions a purer product is obtained if the soap solution is cooled before precipitating the fatty acid.

*mitic* acid. Remove the cake, break it into small pieces, wash it with water by decantation and transfer to a small beaker by means of 95 per cent alcohol. Heat on a water-bath until the palmitic acid is dissolved, then filter through a dry filter paper and allow the filtrate to cool slowly in order to obtain satisfactory crystals. Write the reactions which have taken place in this experiment.

When the palmitic acid has completely crystallized filter off the alcohol, dry the crystals between the filter papers and try the tests given in Experiment 9, below.



FIG. 39—PALMITIC ACID.

8. **Salting-out Experiments.**—To 25 c.c. of soap solution, prepared as described above, add *solid* sodium chloride to the point of saturation, with continual stirring. A menstruum is thus formed in which the soap is insoluble. This salting-out process is entirely analogous to the salting-out of proteins (see page 106).

9. **Palmitic Acid.**—(a) Examine the crystals under the microscope and compare them with those shown in Fig. 39, above.

(b) *Solubility.*—Try the solubility of palmitic acid in the same solvents as used on fats (see page 143).

(c) *Melting-point.*—Determine the melting-point of palmitic acid by one of the methods given on page 146.

(d) *Formation of Transparent Spot on Paper.*—Melt a little of the fatty acid and allow a drop to fall upon a piece of ordinary writing paper. How does this compare with the action of a fat under similar circumstances?

(e) *Acrolein Test.*—Apply the test as given under 4, page 143. Explain the result.

10. **Saponification of Lard.**—To 25 grams of lard in a flask add 75 c.c. of alcoholic-potash solution and warm upon a water-bath until saponification is complete. (This point is indicated by the complete solubility of a drop of the solution when allowed to fall into a little water.) Now transfer the solution from the flask to an evaporating dish containing about 100 c.c. of water and heat on a water-bath until all the alcohol has been driven off. Precipitate the fatty acid with hydrochloric acid and cool the solution. Remove the fatty acid which rises to the surface, neutralize the solution with sodium carbonate and evaporate to dryness. Extract the residue with alcohol, remove the alcohol by evaporation upon a water-bath and on the residue of glycerol thus obtained make the tests as given below.

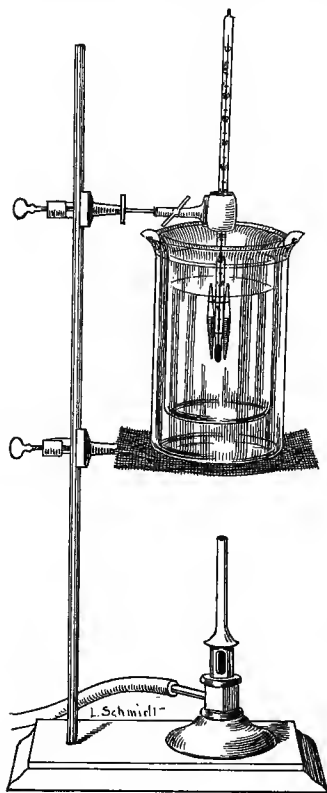


FIG. 40.—MELTING-POINT APPARATUS.

glycerol to this suspended precipitate and note what occurs.

12. **Melting-Point of fat.** *First Method.*—Insert one of the melting-point tubes, furnished by the instructor, into the liquid fat and draw up the fat until the bulb of the tube is about one-half full of the material. Then fuse one end of the tube in the flame of a bunsen burner and fasten the tube to a thermometer by means of a rubber band in such a manner that the bottom of the fat column is on a level with the bulb of the thermometer (Fig. 40, above). Fill a beaker of medium size about two-thirds full of water and place it within a second larger beaker which also contains water, the two vessels being separated by pieces of cork.

11. **Glycerol.** (a) *Taste.*—What is the taste of glycerol?

(b) *Solubility.*—Try the solubility of glycerol in water, alcohol and ether.

(c) *Acrolein Test.*—Repeat the test as given under 4, page 143.

(d) *Borax Fusion Test.*—Fuse a little glycerol on a platinum wire with some powdered borax and note the characteristic green flame. This color is due to the glycerol ester of boric acid.

(e) *Fehling's Test.*—How does this result compare with the results on the sugars?

(f) *Solution of  $\text{Cu}(\text{OH})_2$ .*—Form a little cupric hydroxide by mixing copper sulphate and potassium hydroxide. Add a little



Immerse the bulb of the thermometer and the attached tube in such a way that the bulb is about midway between the upper and the lower surfaces of the water of the inner beaker. The upper end of the tube being open it must extend above the surface of the surrounding water. Apply gentle heat, stir the water, and note the temperature at which the fat first begins to melt. This point is indicated by the initial transparency. For ordinary fats, raise the temperature very cautiously from 30° C. To determine the *congealing-point* remove the flame and note the temperature at which the fat begins to solidify. Record the melting- and congealing-points of the various fats submitted by the instructor.

*Second Method.*—Fill a small evaporating dish about one-half full of mercury and place it on a water-bath. Put a small drop of the fat under examination on an ordinary cover glass and place this upon the surface of the mercury. Raise the temperature of the water-bath slowly and by means of a thermometer whose bulb is immersed in the mercury, note the melting-point of the fat. Determine the congealing-point by removing the flame and leaving the fat drop and coverglass in position upon the mercury. How do the melting-points as determined by this method compare with those as determined by the first method? Which method is the more accurate, and why?

## CHAPTER VIII.

### PANCREATIC DIGESTION.

As soon as the food mixture leaves the stomach it comes into intimate contact with the bile and the pancreatic juice. Since these fluids are alkaline in reaction there can obviously be no further peptic activity after they have become intimately mixed with the chyme and have neutralized the acidity previously imparted to it by the hydrochloric acid of the gastric juice. The pancreatic juice reaches the intestine through the duct of Wirsung which opens into the intestine near the pylorus.

Normally the secretion of pancreatic juice is brought about by the stimulation produced by the acid chyme as it enters the duodenum. Therefore, any factor which produces an increased flow of gastric juice such, for example, as water<sup>1</sup> will cause a stimulation of the pancreatic secretion. The secretion of pancreatic juice is probably not due to a nervous reflex as was believed by Pawlow but rather, as Bayliss and Starling have shown, is dependent upon the presence, in the epithelial cells of the duodenum and jejunum of a body known as *prosecretin*. This body is changed into *secretin* through the hydrolytic action of the acid present in the chyme. The secretin is then absorbed by the blood, passes to the pancreas and stimulates the pancreatic cells, causing a flow of pancreatic juice. The quantity of juice secreted under these conditions is proportional to the amount of secretin present. The activity of secretin solutions is not diminished by boiling, hence the body does not react like an enzyme. Further study of the body may show it to be a definite chemical individual of relatively low molecular weight. It has not been possible thus far to obtain secretin from any tissues except the mucous membrane of the duodenum and jejunum.

This *secretin* mentioned above belongs to the class of substances called *hormones* or chemical messengers. These hormones play a very important part in the coordination of the activities of certain functions and glands. Other important hormones are those elaborated by the thyroids, the adrenals, the pituitary body (hypophysis), the embryo and the reproductive glands. It is claimed that all active organs of the body produce hormones.

The juice as obtained from a permanent fistula differs greatly in

<sup>1</sup> See chapter on Gastric Digestion.

its properties from the juice as obtained from a temporary fistula, and neither form of fluid possesses the properties of the normal fluid. Pancreatic juice collected by Glaessner from a natural fistula has been found to be a colorless, clear, strongly alkaline fluid which foams readily. It is further characterized by containing albumin, globulin, proteose, and peptone; nucleoprotein is also present in traces.<sup>1</sup> The average daily secretion of pancreatic juice is 650 c.c. and its specific gravity is 1.008. The fluid contains 1.3 per cent of solid matter and the freezing-point is  $-0.47^{\circ}$  C. The normal pancreatic secretion contains at least four distinct enzymes. They are *trypsin*, a proteolytic enzyme; *pancreatic amylase* (amylopsin), an amylytic enzyme; *pancreatic lipase* (steapsin), a fat-splitting enzyme; and *pancreatic rennin*, a milk-coagulating enzyme. *Lactase*, the lactose-splitting enzyme, is also present at certain times.

The most important of the four enzymes of the pancreatic juice is the proteolytic enzyme trypsin. This enzyme resembles pepsin in so far as each has the power of breaking down protein material, but the trypsin has much greater digestive power and is able to cause a more complete decomposition of the complex protein molecule. In the process of normal digestion the protein constituents of the diet are for the most part transformed into proteoses (albumoses) and peptones before coming in contact with the enzyme trypsin. This is not absolutely essential however, since trypsin possesses digestive activity sufficient to transform unaltered native proteins and to produce from their complex molecules comparatively simple fragments. Among the products of tryptic digestion are *proteoses*, *peptones*, *peptides*, *leucine*, *tyrosine*, *aspartic acid*, *glutamic acid*, *alanine*, *phenylalanine*, *glycocoll*, *cystine*, *serine*, *valine*, *proline*, *oxyproline*, *isoleucine*, *arginine*, *lysine*, *histidine*, and *tryptophane*. (The crystalline forms of many of these products are reproduced in Chapter IV.) Trypsin does not occur preformed in the gland, but exists there as a zymogen called *trypsinogen* which bears the same relation to trypsin that pepsinogen does to pepsin. Trypsin has never been obtained in a pure form and therefore very little can be stated definitely as to its nature. The enzyme is the most active in alkaline solution but is also active in neutral or slightly acid solutions. Trypsin is destroyed by mineral acids and may also be destroyed by comparatively weak alkali (2 per cent sodium carbonate) if left in contact for a sufficiently long time. Trypsinogen, on the other hand, is more resistant to the action of alkalis. In pancreatic digestion the protein does not swell as is the case in gastric digestion, but becomes more or less "honey-combed" and it finally disintegrates.

The presence of active *pepsin* in the contents of the intestine has been

<sup>1</sup> Glaessner: *Zeitschrift für physiologische Chemie*, 40, 476, 1904.

demonstrated very recently by Abderhalden and Meyer.<sup>1</sup> It may possibly be that pepsin may play a part in the profound intestinal proteolysis which has up to this time been assigned to trypsin and erepsin (see chapter on Gastric Digestion).

The pancreatic juice which is collected by means of a fistula possesses practically no power to digest protein matter. A body called *enterokinase* occurs in the intestinal juice and has the power of converting trypsinogen into trypsin. This process is known as the "activation" of trypsinogen and through it a juice which is incapable of digesting protein may be made active. Enterokinase is not always present in the intestinal juice since it is secreted only after the pancreatic juice reaches the intestine. It resembles the enzymes in that its activity is destroyed by heat, but differs materially from this class of bodies in that a certain quantity is capable of activating only a definite quantity of trypsinogen. It is, however, generally classified as an enzyme. Enterokinase has been detected in the higher animals, and a kinase possessing similar properties has been shown to be present in bacteria, fungi, impure fibrin, lymph glands, and snake-venom. Mendel and Rettger<sup>2</sup> and others have demonstrated that activation of trypsinogen into trypsin may be brought about in the gland as well as in the intestine of the living organism. The manner of the activation in the gland and the nature of the body causing it are unknown at present. Prym<sup>3</sup> denies that such an activation occurs.

Delezenne claims that trypsinogen may be activated by soluble *calcium salts*. He reports experiments which indicate that proteolytically inactive pancreatic juice, obtained directly from the duct, when treated with salts of this character, assumes the property of digesting protein material. This process by which the trypsinogen is activated through the instrumentality of calcium salts is very rapid and is designated by Delezenne as an "explosion." The recent suggestion of Mays that there may possibly be several precursors of trypsin one of which is activated by enterokinase and the others by other agents, is of interest in this connection.

*Pancreatic amylase (amyllopsin)*, the second of the pancreatic enzymes, is an amylolytic enzyme which possesses somewhat greater digestive power than the salivary amylase (ptyalin) of the saliva. As its name implies, its activity is confined to the starches, and the products of its amylolytic action are dextrans and sugars. The sugars are principally iso-maltose and maltose and these by the further action of an inverting enzyme are partly transformed into dextrose.

<sup>1</sup> Abderhalden and Meyer: *Zeit. physiol. Chem.*, 74, 67, 1911.

<sup>2</sup> Mendel and Rettger: *American Journal of Physiology*, 7.

<sup>3</sup> Prym: *Pflüger's Archiv.*, 104 and 107.

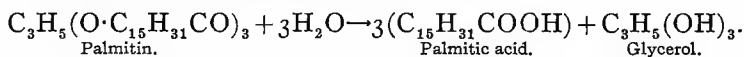
It is possible that the saliva as a digestive fluid is not absolutely essential. The salivary amylase (ptyalin) is destroyed by the hydrochloric acid of the gastric juice and is therefore inactive when the chyme reaches the intestine. Should undigested starch be present at this point however, it would be quickly transformed by the active pancreatic amylase. This enzyme is not present in the pancreatic juice of infants during the first few weeks of life, thus showing very clearly that a starchy diet is not normal for this period.

The pronounced influence of electrolytes upon the action of pancreatic amylase and other amylases has been demonstrated many times.<sup>1</sup> In this connection Bierry<sup>2</sup> has very recently shown that the removal of electrolytes from pancreatic juice by dialysis yields a juice which possesses *no power to split starch*. He further claims that the Cl or Br ion is "absolutely essential to the activity of animal amylases." It is generally recognized that the presence of the Cl ion *facilitates* amylolytic action.<sup>3</sup>

It has been claimed that pancreatic amylase has a slight digestive action upon *unboiled* starch.

The extent to which amylase is present in the feces has been taken as the index of pancreatic activity.

The third enzyme of the pancreatic juice is called *pancreatic lipase* (*steapsin*) and is a fat-splitting enzyme. It has the power of splitting the neutral fats of the food by hydrolysis, into fatty acid and glycerol. A typical reaction would be as follows:



Recent researches make it probable that fats undergo saponification to a certain extent prior to their absorption. The fatty acids formed, in part unite with the alkalis of the pancreatic juice and intestinal secretion to form soluble soaps; in part they are doubtless absorbed dissolved in the bile. Some observers believe that the fats may also be absorbed in emulsion—a condition promoted by the presence of the soluble soaps. After absorption the fatty acids are re-synthesized to form neutral fats with glycerol.

It has been demonstrated that lipase acts best in dilution.<sup>4</sup> This fact is of importance when considered in connection with the fact that ingested fat is better utilized in the human organism when large volumes of water (1000 c.c.) are taken with meals.<sup>5</sup>

<sup>1</sup> For the literature see Kendall and Sherman: *Jour. Am. Chem. Soc.*, 32, 1087, 1910.

<sup>2</sup> Bierry: *Biochem. Zeit.*, 40, 357, 1912.

<sup>3</sup> Wohlgemuth: *Biochem. Zeit.*, 9, 10, 1908; and Kendall and Sherman: *Jour. Am. Chem. Soc.*, 32, 1087, 1910.

<sup>4</sup> Bradley: *Jour. Biol. Chem.*, 8, 251, 1910.

<sup>5</sup> Mattill and Hawk: *Jour. Am. Chem. Soc.*, 33, 1978, 1911.

Pancreatic lipase is very unstable and is easily rendered inert by the action of acid. For this reason it is not possible to prepare an extract having a satisfactory fat-splitting power from a pancreas which has been removed from the organism for a sufficiently long time to have become acid in reaction.

The fourth enzyme of the pancreatic juice is called *pancreatic rennin*. It is a milk-coagulating enzyme whose action is very similar to that of the enzyme *gastric rennin* found in the gastric juice. It is supposed to show its greatest activity at a temperature varying from 60° to 65° C.

The enzymes of the intestinal juice (*succus entericus*) are of great importance to the animal organism. These enzymes include *erepsin*, *sucrase*, *maltase*, *lactase*, and *enterokinase*. According to Boldyreff lipase is also present.

Erepsin is a proteolytic enzyme which has the property of acting upon the proteoses, peptones and peptides which are formed through the action of trypsin and further splitting them into *amino acids*. Erepsin has no power of digesting any native proteins except caseinogen, histones, and protamines. It possesses its greatest activity in an alkaline solution although it is slightly active in acid solution. An extract of the intestinal erepsin may be prepared by treating the finely divided intestine of a *cat*, *dog*, or *pig* with toluol- or chloroform-water and permitting the mixture to stand with occasional shaking for 24-72 hours.<sup>1</sup> Enzymes similar to erepsin occur in various tissues of the organism.

In cases of gastric cancer a peptide-splitting enzyme is present in the stomach contents. The glycyl-tryptophane test is used for its detection (see chapters on Enzymes and Gastric Digestion).

The three invertases *sucrase*, *maltase*, and *lactase* are also important enzymes of the intestinal mucosa. The sucrase acts upon sucrose and inverts it with the formation of *invert sugar* (dextrose and lævulose). Some investigators claim that sucrase is also present in saliva and gastric juice. It probably does not exist normally in either of these digestive juices, however, and if found owes its presence to the excretory processes of certain bacteria. Sucrases may also be obtained from several vegetable sources. For investigational purposes it is ordinarily obtained from yeast (see p. 13). It exhibits its greatest activity in the presence of a slight acidity but if the acidity be increased to any extent the reaction is inhibited.

Lactase is an enzyme which inverts lactose with the consequent formation of dextrose and galactose. Its action is entirely analogous, in type, to that of sucrase. It has apparently been proven that lactase occurs in the intestinal mucosa of the young of all animals which suckle

<sup>1</sup> See page 15.

their offspring.<sup>1</sup> It may also occur in the intestinal mucosa of certain adult animals if such animals be maintained upon a ration containing more or less lactose. Fischer and Armstrong have demonstrated the reversible action<sup>2</sup> of lactase.

For discussions of *maltase* and *enterokinase* see pages 62 and 150 respectively.

### PREPARATION OF AN ARTIFICIAL PANCREATIC JUICE.<sup>3</sup>

After removing the fat from the pancreas of a pig or sheep, finely divide the organ by means of scissors and grind it in a mortar. If convenient, the use of an ordinary meat chopper is a very satisfactory means of preparing the pancreas.

When finely divided as above the pancreas should be placed in a 500 c.c. flask, about 150 c.c. of 30 per cent alcohol added and the flask and contents shaken frequently for twenty-four hours. (What is the reaction of this alcoholic extract at the end of this period, and why?) Strain the alcoholic extract through cheese cloth, filter, nearly neutralize with potassium hydroxide solution and then exactly neutralize it with 0.5 per cent sodium carbonate.

### PRODUCTS OF TRYPTIC DIGESTION.

Take about 200 grams of lean beef which has been freed from fat and finely ground and place it in a large-sized beaker. Introduce equal volumes of the pancreatic extract prepared as above and 0.5 per cent sodium carbonate, add 5 c.c. of an alcoholic solution of thymol to prevent putrefaction, and place the beaker in an incubator at 40° C. Stir the contents of the beaker frequently and add more thymol if it becomes necessary. Allow digestion to proceed for from 2 to 5 days and then separate the products formed as follows: Strain off the undissolved residue through cheese cloth, nearly neutralize the solution with dilute hydrochloric acid and then exactly neutralize it with 0.2 per cent hydrochloric acid. A precipitate at this point would indicate *alkali metaprotein* (alkali albuminate). Filter off any precipitate and divide the filtrate into two parts, a one-fourth and a three-fourth portion.

Transfer the one-fourth portion to an evaporating dish and make the separation of *proteoses* and *peptones* as well as the final tests upon these bodies according to the directions given on page 120.

Place about 5 c.c. of the three-fourth portion in a test-tube and

<sup>1</sup> Mendel and Mitchell: *American Journal of Physiology*, 20, 81, 1907.

<sup>2</sup> See p. 8.

<sup>3</sup> For other methods of preparation see Karl Mays: *Zeitschrift für physiologische Chemie*, 38, 428, 1903.

add about 1 c.c. of bromine water. A violet coloration indicates the presence of *tryptophane* (see page 82. Also see glycyL-tryptophane reaction in chapter on Enzymes.) Concentrate<sup>1</sup> the remainder of the three-fourth portion to a thin syrup and make the separation of *leucine* and *tyrosine* according to the directions given on page 82.

## GENERAL EXPERIMENTS ON PANCREATIC DIGESTION.

### EXPERIMENTS ON TRYPSIN.

1. **The Most Favorable Reaction for Tryptic Digestion.**—Prepare seven tubes as follows:

(a) 2-3 c.c. of *neutral* pancreatic extract + 2-3 c.c. of water.

(b) 2-3 c.c. of *neutral* pancreatic extract + 2-3 c.c. of 1 per cent sodium carbonate.

(c) 2-3 c.c. of *neutral* pancreatic extract + 2-3 c.c. of 0.5 per cent sodium carbonate.

(d) 2-3 c.c. of *neutral* pancreatic extract + 2-3 c.c. of 0.2 per cent hydrochloric acid.

(e) 2-3 c.c. of *neutral* pancreatic extract + 2-3 c.c. of 0.2 per cent *combined* hydrochloric acid.

(f) 2-3 c.c. of *neutral* pancreatic extract + 2-3 c.c. of 0.4 per cent boric acid.

(g) 2-3 c.c. of *neutral* pancreatic extract + 2-3 c.c. of 0.4 per cent acetic acid.

Add a small piece of fibrin to the contents of each tube and keep them at 40° C. noting the progress of digestion. In which tube do we find the most satisfactory digestion, and why? How do the indications of the digestion of fibrin by trypsin differ from the indications of the digestion of fibrin by pepsin?

2. **The Most Favorable Temperature.**—(For this and the following series of experiments under tryptic digestion use the *neutral* extract plus an *equal volume* of 0.5 per cent sodium carbonate.) In each of four tubes place 5 c.c. of *alkaline* pancreatic extract. Immerse one tube in cold water from the faucet, keep a second at room temperature and place a third in the incubator or water-bath at 40° C. Boil the contents of the fourth for a few moments, then cool and also keep it at 40° C. Into each tube introduce a small piece of fibrin and note the progress of digestion. In which tube does the most rapid digestion occur? What is the reason?

3. **Influence of Metallic Salts, Etc.**—Prepare a series of tubes and into each tube place 6 volumes of water, 3 volumes of alkaline pancre-

<sup>1</sup> If the solution is alkaline in reaction, while it is being concentrated, the amino acids will be broken down and ammonia will be liberated.



atic extract and 1 volume of one of the chemicals listed in Experiment 18 under Salivary Digestion, page 66.

Introduce a small piece of fibrin into each of the tubes and keep them at 40° C. for one-half hour. Shake the tubes frequently. In which tubes do we get the least digestion?

4. **Influence of Bile.**—Prepare five tubes as follows:

- (a) Five c.c. of pancreatic extract +  $1/2$ -1 c.c. of bile.
- (b) Five c.c. of pancreatic extract + 1-2 c.c. of bile.
- (c) Five c.c. of pancreatic extract + 2-3 c.c. of bile.
- (d) Five c.c. of pancreatic extract + 5 c.c. of bile.
- (e) Five c.c. of pancreatic extract.

Introduce into each tube a small piece of fibrin and keep them at 40° C. Shake the tubes frequently and note the progress of digestion. Does the presence of bile retard tryptic digestion? How do these results agree with those obtained under gastric digestion?

#### EXPERIMENTS ON PANCREATIC AMYLASE.

1. **The Most Favorable Reaction.**—Prepare seven tubes as follows:

- (a) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of water.
- (b) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 1 per cent sodium carbonate.
- (c) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.5 per cent sodium carbonate.
- (d) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.2 per cent hydrochloric acid.
- (e) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.2 per cent *combined* hydrochloric acid.
- (f) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.4 per cent boric acid.
- (g) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.4 per cent acetic acid.

Shake each tube thoroughly and place them in the incubator or water-bath at 40° C. At the end of a half-hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Fehling's test. Where do you find the most satisfactory digestion? How do the results compare with those obtained from the similar series under Trypsin, page 154?

2. **The Most Favorable Temperature.**—(For this and the following series of experiments upon pancreatic amylase use the *neutral* extract plus an equal volume of 0.5 per cent sodium carbonate.) In

each of four tubes place 2-3 c.c. of *alkaline* pancreatic extract. Immerse one tube in cold water from the faucet, keep a second at room temperature, and place a third on the water-bath at 40° C. Boil the contents of the fourth for a few moments, then cool and also keep it at 40° C. Into each tube introduce 2-3 c.c. of starch paste and note the progress of digestion. At the end of one-half hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Fehling's test. In which tube do you find the most satisfactory digestion? How does this result compare with the result obtained in the similar series of experiments under Trypsin (see page 154)?

3. **Influence of Metallic Salts, etc.**—Prepare a series of tubes and into each place 3 volumes of water, 3 volumes of *alkaline* pancreatic extract, 1 volume of one of the chemicals listed in Experiment 18 under Salivary Digestion, page 66, and 3 volumes of starch paste. Be sure to introduce the *starch paste* into the tube *last*. Why? Shake the tubes well and place them in the incubator or water-bath at 40° C. At the end of a half-hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Fehling's test. What are your conclusions?

4. **Influence of Bile.**—Prepare five tubes as follows:

(a) 2-3 c.c. of pancreatic extract + 2-3 c.c. of starch paste + 1/2-1 c.c. of bile.

(b) 2-3 c.c. of pancreatic extract + 2-3 c.c. of starch paste + 1-2 c.c. of bile.

(c) 2-3 c.c. of pancreatic extract + 2-3 c.c. of starch paste + 2-3 c.c. of bile.

(d) 2-3 c.c. of pancreatic extract + 2-3 c.c. of starch paste + 5 c.c. of bile.

(e) 2-3 c.c. of pancreatic extract + 2-3 c.c. of starch paste.

Shake the tubes thoroughly and place them in the incubator or water-bath at 40° C. Note the progress of digestion frequently and at the end of a half-hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Fehling's test. What are your conclusions regarding the influence of bile upon the action of pancreatic amylase?

5. **Digestion of Dry Starch.**—To a little *dry* starch in a test-tube add about 5 c.c. of pancreatic extract and place the tube in the incubator or water-bath at 40° C. At the end of a half-hour filter and test separate portions of the filtrate by the iodine and Fehling tests. What do you conclude regarding the action of pancreatic amylase upon dry starch? Compare this result with that obtained in the similar experiment under Salivary Digestion (page 65).

6. **Digestion of Inulin.**—To 5 c.c. of inulin solution in a test-tube add 10 drops of pancreatic extract and place the tube in the incubator or water-bath at 40° C. After one-half hour test the solution by Fehling's test.<sup>1</sup> Is any reducing substance present? What do you conclude regarding the digestion of inulin by pancreatic amylase?

#### EXPERIMENTS ON PANCREATIC LIPASE.

1. **"Litmus-milk" Test.**—Into each of two test-tubes introduce 10 c.c. of milk and a small amount of litmus powder. To the contents of one tube add 3 c.c. of *neutral* pancreatic extract and to the contents of the other tube add 3 c.c. of water or of *boiled* neutral pancreatic extract. Keep the tubes at 40° C. and note any changes which may occur. What is the result and how do you explain it?

2. **Ethyl Butyrate Test.**—Into each of two test-tubes introduce 4 c.c. of water, 2 c.c. of ethyl butyrate,  $C_3H_7COO.C_2H_5$ , and a small amount of litmus powder. To the contents of one tube add 4 c.c. of *neutral* pancreatic extract and to the contents of the other tube add 4 c.c. of water or of *boiled* neutral pancreatic extract. Keep the tubes at 40° C. and observe any changes which may occur. What is the result and how do you explain it? Write the equation for the reaction which has taken place.

#### EXPERIMENTS ON PANCREATIC RENNIN.

Prepare four test-tubes as follows:

- (a) Five c.c. of milk + 10 drops of *neutral* pancreatic extract.
- (b) Five c.c. of milk + 20 drops of *neutral* pancreatic extract.
- (c) Five c.c. of milk + 10 drops of *alkaline* pancreatic extract.
- (d) Five c.c. of milk + 20 drops of *alkaline* pancreatic extract.

Place the tubes at 60°–65° C. for a half hour *without shaking*. Note the formation of a clot.<sup>2</sup> How does the action of pancreatic rennin compare with the action of the gastric rennin?

<sup>1</sup> If the inulin solution gives a reduction before being acted upon by the pancreatic juice, it will be necessary to determine the extent of the original reduction by means of a "check" test (see page 52).

<sup>2</sup> This reaction will not always succeed, owing to conditions which are not well understood.

## CHAPTER IX.

### BILE.

The bile is secreted continuously by the liver and passes into the intestine through the common bile duct which opens near the pylorus. Bile is *not* secreted continuously *into the intestine*. In a fasting animal no bile enters the intestine, but when food is taken the bile begins to flow; the length of time elapsing between the ingestion of the food and the secretion of the bile as well as the qualitative and quantitative characteristics of the secretion depending upon the nature of the food ingested. Fats, the extractives of meat and the protein end-products of gastric digestion (proteoses and peptones), cause a copious secretion of bile, whereas such substances as water, acids and boiled starch paste fail to do so. In general a rich protein diet is supposed to increase the amount of bile secreted, whereas a carbohydrate diet would cause a much less decided increase and might even tend to decrease the amount. It has been demonstrated by Bayliss and Starling that the secretion of bile is under the control of the same mechanism that regulates the flow of pancreatic juice (see p. 148). In other words, the hydrochloric acid of the chyme, as it enters the duodenum transforms prosecretin into secretin and this in turn enters the circulation, is carried to the liver, and stimulates the bile-forming mechanism to increased activity.

We may look upon the bile as an *excretion* as well as a *secretion*. In the fulfillment of its excretory function it passes such bodies as lecithin, metallic substances, cholesterol, and the decomposition products of hæmoglobin into the intestine and in this way aids in removing them from the organism. The bile assists materially in the absorption of fats from the intestine by its solvent action on the fatty acids formed by the action of the pancreatic juice.

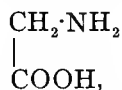
The bile is aropy, viscid substance which is alkaline in reaction to litmus,<sup>1</sup> and ordinarily possesses a decidedly bitter taste. It varies in color in the different animals, the principal variations being yellow, brown, and green. Fresh human bile from the living organism ordinarily has a green or golden-yellow color. Postmortem bile is variable in color. It is very difficult to determine accurately the amount of normal bile secreted during any given period. For an adult man it has been variously estimated at from 500 c.c. to 1100 c.c. for twenty-

<sup>1</sup> It does not contain any *free hydroxyl ions*, however.

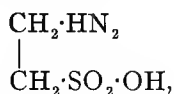
four hours. The specific gravity of the bile varies between 1.010 and 1.040, and the freezing-point is about  $-0.56^{\circ}$  C. As secreted by the liver, the bile is a clear, limpid fluid which contains a relatively low content of solid matter. Such bile would have a specific gravity of approximately 1.010. After it reaches the gall-bladder, however, it becomes mixed with mucous material from the walls of the gall-bladder, and this process coupled with the continuous absorption of water from the bile has a tendency to concentrate the secretion. Therefore the bile as we find it in the gall-bladder, ordinarily possesses a higher specific gravity than that of the freshly secreted fluid. The specific gravity under these conditions may run as high as 1.040.

The principal constituents of the bile are the *salts of the bile acids, bile pigments, neutral fats, lecithin, phosphatides, and cholesterol*, besides the salts of *iron, copper, calcium, and magnesium*. *Zinc* has also frequently been found in traces.

The bile acids, which are elaborated exclusively by the hepatic cells, may be divided into two groups, the *glycocholic acid* group and the *taurocholic acid* group. In human bile glycocholic acid predominates, while taurocholic acid is the more abundant in the bile of carnivora. The bile acids are conjugate *amino-acids*, the glycocholic acid yielding *glycocol*,



and *cholic acid* upon decomposition, whereas taurocholic acid gives rise to *taurine*,



and *cholic acid* under like conditions. Glycocholic acid contains some nitrogen but no sulphur, whereas taurocholic acid contains both these elements. The sulphur of the taurocholic acid is present in the taurine (amino-ethyl-sulphonic-acid), of which it is a characteristic constituent. There are several varieties of cholic acid and therefore we have several forms of glycocholic and taurocholic acids, the variation in constitution depending upon the nature of the cholic acid which enters into the combination. The bile acids are present in the bile as salts of one of the alkalis, generally sodium. The sodium glycocholate and sodium taurocholate may be isolated in crystalline form, either as balls or rosettes of fine needles or in the form of prisms having ordinarily four or six sides (Fig. 41, p. 160). The salts of the bile acids are dextro-rotatory.

Among other properties these salts have the power of holding the cholesterol and lecithin of the bile in solution.

Hammarsten has demonstrated a third group of bile acids in the bile of the shark. This same group very probably occurs in certain other animals also. These acids are very rich in sulphur and resemble ethereal sulphuric acids inasmuch as upon treatment with boiling hydrochloric acid they yield sulphuric acid.



FIG 41.—BILE SALTS.

The bile pigments are important and interesting biliary constituents. The following have been isolated: *bilirubin*, *biliverdin*, *bilifuscin*, *biliprasin*, *bilihumin*, *bilicyanin*, *choleprasin*, and *choletelin*. Of these, bilirubin and biliverdin are the most important and predominate in normal bile. The colors possessed by the various varieties of normal bile are due almost entirely to these two pigments, the biliverdin being the predominant pigment in greenish bile and the bilirubin being the principal pigment in lighter colored bile. The pigments, other than the two just mentioned, have been found almost exclusively in biliary calculi or in altered bile obtained as post-mortem examinations.

Bilirubin, which is perhaps the most important of the bile pigments, is apparently derived from the blood pigment, the iron freed in the process being held in the liver. Bilirubin has the same percentage composition as hæmatoporphyrin, which may be produced from hæmatin. It is a specific product of the liver cells, but may also be formed in other parts of the body. The pigment may be isolated in the form of a reddish-yellow powder or may be obtained in part, in the form of reddish-yellow rhombic plates (Fig. 42, p. 161) upon the spontaneous evaporation of its chloroform solution. The crystalline form of bilirubin is practically the same as that of hæmatoidin. It is easily soluble in chloroform,

somewhat less soluble in alcohol and only slightly soluble in ether and benzene. Bilirubin has the power of combining with certain metals, particularly calcium, to form combinations which are no longer soluble in the solvents of the unaltered pigment. Upon long standing in contact with the air, the reddish-yellow bilirubin is oxidized with the formation of the green biliverdin. Bilirubin occurs in animal fluids as soluble bilirubin-alkali.



FIG. 42.—BILIRUBIN (HÆMATOIDIN). (Ogden.)

Solutions of bilirubin exhibit no absorption-bands. If an ammoniacal solution of bilirubin-alkali in water is treated with a solution of zinc chloride, however, it shows bands similar to those of bilicyanin (Absorption Spectra, Plate II), the two bands between C and D being rather well defined.

Biliverdin is particularly abundant in the bile of herbivora. It is soluble in alcohol and glacial acetic acid and insoluble in water, chloroform, and ether. Biliverdin is formed from bilirubin upon oxidation. It is an amorphous substance, and in this differs from bilirubin which may be at least partly crystallized under proper conditions. Biliverdin may be obtained in the form of a green powder. In common with bilirubin, it may be converted into hydrobilirubin by nascent hydrogen.

The neutral solution of bilicyanin or cholecyanin is bluish-green or steel-blue and possesses a blue fluorescence, the alkaline solution is green with no appreciable fluorescence and the strongly acid solution is violet-blue. The alkaline solution exhibits three absorption-bands, the first a dark, well-defined band between C and D, somewhat nearer C; the second a less sharply-defined band extending across D and the third a rather faint band between E and F, near E (Absorption Spectra, Plate II). The strongly acid solution exhibits two absorption bands, both lying between C and E and separated by a narrow space near D. A third band, exceedingly faint, may ordinarily be seen between b and F.

*Biliary calculi*, otherwise designated as *biliary concretions* or *gall stones*, are frequently formed in the gall-bladder. These deposits may

be divided into three classes, *cholesterol calculi*, *pigment calculi*, and calculi made up almost entirely of *inorganic material*. This last class of calculus is formed principally of the carbonate and phosphate of calcium and is rarely found in man although quite common to cattle. The pigment calculus is also found in cattle, but is more common to man than the inorganic calculus. This pigment calculus ordinarily consists principally of bilirubin in combination with calcium; biliverdin is sometimes found in small amount. The cholesterol calculus is the one found most frequently in man. These may be formed almost entirely of cholesterol, in which event the color of the calculus is very light, or they may contain more or less pigment and inorganic matter mixed with the cholesterol, which tends to give us calculi of various colors.

For discussion of cholesterol see page 270.

#### EXPERIMENTS ON BILE.

1. **Reaction.**—Test the reaction of fresh ox bile to litmus, phenolphthalein and congo red.

2. **Nucleoprotein.**—Acidify a small amount of bile with dilute acetic acid. A precipitate of nucleoprotein forms. Bile acids will also precipitate here under proper conditions of acidity.

3. **Inorganic Constituents.**—Test for chlorides, sulphates, and phosphates (see page 64).

4. **Tests for Bile Pigments.** (a) *Gmelin's Test.*—To about 5 c.c. of *concentrated* nitric acid in a test-tube add 2–3 c.c. of diluted bile *carefully* so that the two fluids do not mix. At the point of contact note the various colored rings, green, blue, violet, red and reddish-yellow. Repeat this test with different dilutions of bile and observe its delicacy.

(b) *Rosenbach's Modification of Gmelin's Test.*—Filter 5 c.c. of diluted bile through a small filter paper. Introduce a drop of *concentrated* nitric acid into the cone of the paper and note the succession of colors as given in Gmelin's test.

(c) *Nakayama's Reaction.*—To 5 c.c. of diluted bile in a test-tube add an equal volume of a 10 per cent solution of barium chloride, centrifugate the mixture, pour off the supernatant fluid, and heat the precipitate with 2 c.c. of Nakayama's reagent.<sup>1</sup> In the presence of bile pigments the solution assumes a blue or green color.

(d) *Huppert's Reaction.*—Thoroughly shake equal volumes of undiluted bile and milk of lime in a test-tube. The pigments unite with the calcium and are precipitated. Filter off the precipitate, wash it with water, and

<sup>1</sup> Prepared by combining 99 c.c. of alcohol and 1 c.c. of *fuming* hydrochloric acid containing 4 grams of ferric chloride per liter.



transfer to a small beaker. Add alcohol acidified slightly with hydrochloric acid and warm upon a water-bath until the solution becomes colored an emerald green.

In examining urine for bile pigments, according to Steensma, this procedure may give negative results even in the presence of the pigments, owing to the fact that the acid-alcohol is not a sufficiently strong oxidizing agent. He therefore suggests the addition of a drop of a 0.5 per cent solution of sodium nitrite to the acid-alcohol mixture before warming on the water-bath. Try this modification also.

(e) *Hammarsten's Reaction*.—To about 5 c.c. of Hammarsten's reagent<sup>1</sup> in a small evaporating dish add a few drops of diluted bile. A green color is produced. If more of the reagent is now added the play of colors as observed in Gmelin's test may be obtained.

(f) *Smith's Test*.—To 2–3 c.c. of diluted bile in a test-tube add carefully about 5 c.c. of dilute tincture of iodine (1:10) so that the fluids do not mix. A play of colors, *green, blue and violet*, is observed. In making this test upon the urine ordinarily only the *green* color is observed.

(g) *Salkowski-Schippers Reaction*.—To 10 c.c. of diluted bile in a test-tube add 5 drops of a 20 per cent solution of sodium carbonate and 10 drops of a 20 per cent solution of calcium chloride. Filter off the resultant precipitate upon a hardened filter-paper and wash it with water. Remove the precipitate to a small porcelain dish, add 3 c.c. of an acid-alcohol mixture<sup>2</sup> and a few drops of a dilute solution of sodium nitrite and heat. The production of a green color indicates the presence of bile pigments.

(h) *Bonanno's Reaction*.<sup>3</sup>—Place 5–10 c.c. of diluted bile in a small porcelain evaporating dish and add a few drops of Bonanno's reagent.<sup>4</sup> An emerald-green color will develop.

**5. Tests for Bile Acids.** (a) *Pettenkofer's Test*.—To 5 c.c. of diluted bile in a test-tube add 5 drops of a 5 per cent solution of sucrose. Now run about 2–3 c.c. of concentrated sulphuric acid *carefully* down the side of the tube and note the *red* ring at the point of contact. Upon slightly agitating the contents of the tube the whole solution gradually assumes a *reddish* color. As the tube becomes warm, it should be cooled in running water in order that the temperature of the solution may not rise above 70° C.

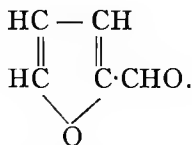
<sup>1</sup> Hammarsten's reagent is made by mixing 1 volume of 25 per cent nitric acid and 19 volumes of 25 per cent hydrochloric acid and then adding 1 volume of this acid mixture to 4 volumes of 95 per cent alcohol.

<sup>2</sup> Made by adding 5 c.c. of concentrated hydrochloric acid to 95 c.c. of 96 per cent alcohol.

<sup>3</sup> *Il Tommasi*, 2, No. 21.

<sup>4</sup> This reagent may be prepared by dissolving 2 grams of sodium nitrite in 100 c.c. of concentrated hydrochloric acid.

(b) *Mylius's Modification of Pettenkofer's Test.*—To approximately 5 c.c. of diluted bile in a test-tube add 3 drops of a very dilute (1:1000) aqueous solution of furfural,



Now run about 2–3 c.c. of concentrated sulphuric acid carefully down the side of the tube and note the *red* ring as above. In this case, also, upon shaking the tube the whole solution is colored red. Keep the temperature of the solution below 70° C as before..

(c) *Neukomm's Modification of Pettenkofer's Test.*—To a few drops of diluted bile in an evaporating dish add a trace of a dilute sucrose solution and one or more drops of dilute sulphuric acid. Evaporate on a water-bath and note the development of a *violet* color at the edge of the evaporating mixture. Discontinue the evaporation as soon as the color is observed.

(d) *v. Udránsky's Test.*—To 5 c.c. of diluted bile in a test-tube add 3–4 drops of a very dilute (1:1,000) aqueous solution of furfural. Place the thumb over the top of the tube and shake the tube until a thick foam is formed. By means of a small pipette add 2–3 drops of concentrated sulphuric acid to the foam and note the *dark pink* coloration produced.

(e) *Guerin's Reaction.*—To equal volumes of diluted bile and alcohol in a test-tube add 5–6 drops of a *saturated* aqueous solution of furfural and 5–6 drops of concentrated sulphuric acid. A *blue* color indicates bile acids.

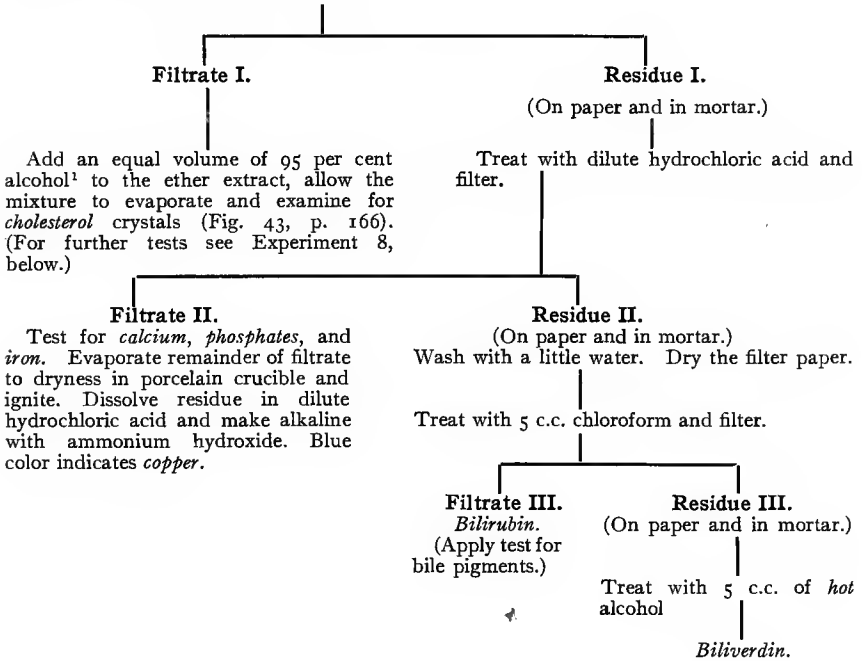
(f) *Hay's Test.*—This test is based upon the principle that bile acids have the property of reducing the surface tension of fluids in which they are contained. The test is performed as follows: Cool about 10 c.c. of diluted bile in a test-tube to 17° C. or lower and sprinkle a little finely pulverized sulphur upon the surface of the fluid. The presence of bile acids is indicated if the sulphur sinks to the bottom of the liquid, the rapidity with which the sulphur sinks depending upon the quantity of bile acids present in the mixture. The test is said to react with bile acids when they are present in the proportion 1:120,000.

Some investigators claim that it is impossible to differentiate between bile acids and bile pigments by this test.

6. **Crystallization of Bile Salts.**—To 25 c.c. of *undiluted* bile in an evaporating dish add enough animal charcoal to form a paste and evaporate to dryness on a water-bath. Remove the residue, grind it in

a mortar, and transfer it to a small flask. Add about 50 c.c. of 95 per cent alcohol and boil on a water-bath for 20 minutes. Filter, and add ether to the filtrate until there is a slight *permanent* cloudiness. Cover the vessel and stand it away until crystallization is complete. Examine the crystals under the microscope and compare them with those shown in Fig. 41, page 160. Try one of the tests for bile acids upon some of the crystals.

7. **Analysis of Biliary Calculi.**—Grind the calculus in a mortar with 10 c.c. of ether. Filter.



8. **Tests for Cholesterol.**

(a) *Microscopical Examination.*—Examine the crystals under the microscope and compare them with those shown in Fig. 43, p. 166.

(b) *Iodine-sulphuric Acid Test.*—Place a few crystals of cholesterol in one of the depressions of a test-tablet and treat with a drop of concentrated sulphuric acid and a drop of a very dilute solution of iodine. A play of colors consisting of *violet, blue, green, and red* results.

(c) *The Liebermann-Burchard Test.*—Dissolve a few crystals of cholesterol in 2 c.c. of chloroform in a dry test-tube. Now add 10 drops of acetic anhydride and 1-3 drops of concentrated sulphuric acid. The solution becomes *red, then blue, and finally bluish-green* in color.

<sup>1</sup> The alcohol is added because of the fact that it is often found that crystallization from pure ether does not yield typical cholesterol crystals.

(d) *Salkowski's Test*.—Dissolve a few crystals of cholesterol in a little chloroform and add an equal volume of concentrated sulphuric acid. A play of colors from *bluish-red* to *cherry-red* and *purple* is noted in the chloroform while the acid assumes a marked green fluorescence.

(e) *Schiff's Reaction*.—To a little cholesterol in an evaporating dish add a few drops of Schiff's reagent.<sup>1</sup> Evaporate to dryness over a low flame and observe the *reddish-violet* residue which changes to a *bluish-violet*.

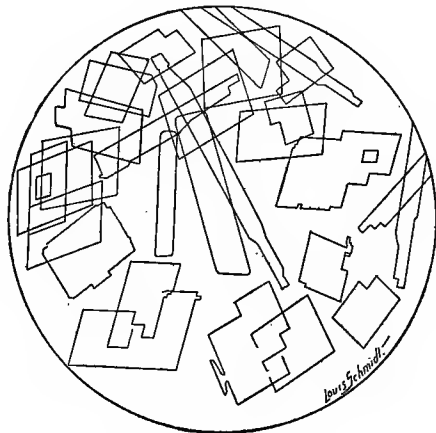


FIG. 43.—CHOLESTEROL.

9. **Preparation of Taurine**.—To 300 c.c. of bile in a casserole add 100 c.c. of hydrochloric acid and heat until a sticky mass (dyslysin) is formed. This point may be determined by drawing out a thread-like portion of the mass by means of a glass rod, and if it solidifies immediately and assumes a brittle character we may conclude that all the taurocholic and glycocholic acid has been decomposed. Decant the solution and concentrate it to a small volume on the water-bath. Filter the hot solution to remove sodium chloride and other substances which may have separated, and evaporate the filtrate to dryness. Dissolve the residue in 5 per cent hydrochloric acid and precipitate with ten volumes of 95 per cent alcohol. Filter off the taurine and recrystallize it from hot water. (Save the alcoholic filtrate for the preparation of glycocoll, below.) Make the following tests upon the taurine crystals.

(a) Examine them under the microscope and compare with Fig. 44.

(b) Heat a crystal upon platinum foil. The taurine at first melts, then turns brown, and finally carbonizes as the temperature is raised. Note the suffocating odor. What is it?

<sup>1</sup> Schiff's reagent consists of a mixture of three volumes of concentrated sulphuric acid and one volume of 10 per cent ferric chloride.

(c) Test the solubility of the crystals in water and in alcohol.

(d) Grind up a crystal with four times its volume of dry sodium carbonate and fuse on platinum foil. Cool the residue, transfer it to a test-tube, and dissolve it in water. Add a little dilute sulphuric acid



FIG. 44.—TAURINE.

and note the odor of hydrogen sulphide. Hold a piece of filter paper, moistened with a *small* amount of lead acetate, over the opening of the test-tube and observe the formation of lead sulphide.

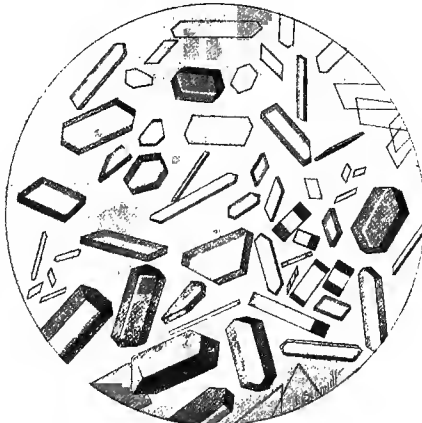


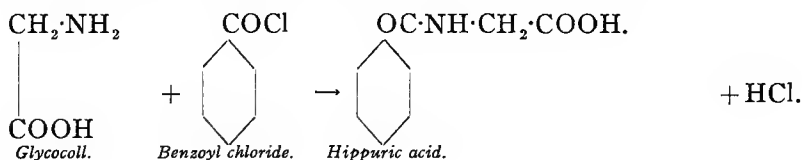
FIG. 45.—GLYCOCOLL.

10. **Preparation of Glycocoll.**—Concentrate the alcoholic filtrate from the last experiment (9) until no more alcohol remains. The glycocoll is present here in the form of an hydrochloride and may be liberated from this combination by the addition of freshly precipitated

lead hydroxide or by lead hydroxide solution. Remove the lead by hydrogen sulphide. Filter and decolorize the filtrate by animal charcoal. Filter again, concentrate the filtrate, and set it aside for crystallization. Glycocoll separates as colorless crystals (Fig. 45.)

II. **Synthesis of Hippuric Acid.**—To some of the glycocoll prepared in the last experiment or furnished by the instructor, add a little water, about 1 c.c. of benzoyl chloride and render alkaline with potassium hydroxide solution. Stopper the tube and shake it until no more heat is evolved. Now render strongly alkaline with potassium hydroxide and shake the mixture until no odor of benzoyl chloride can be detected. Cool, acidify with hydrochloric acid, add an equal volume of petroleum ether, and shake thoroughly to remove the benzoic acid. (Evaporate this solution and note the crystals of benzoic acid. Compare them with those shown in Fig. 99, page 308.) Decant the ethereal solution into a porcelain dish and extract again with ether. The hippuric acid remains in the aqueous solution. Filter it off and wash it with a small amount of cold water while still on the filter. Remove it to a small, shallow vessel, dissolve it in a small amount of hot water and set it aside for crystallization. Examine the crystals microscopically and compare them with those in Fig. 97, page 300.

The chemistry of the synthesis is represented thus:

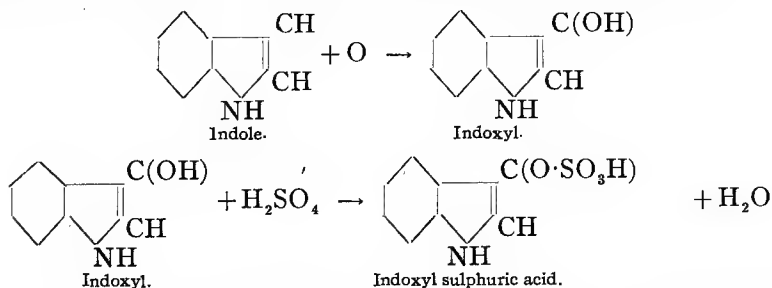


## CHAPTER X.

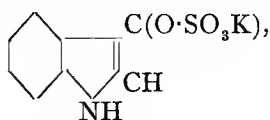
### PUTREFACTION PRODUCTS.

The putrefactive processes in the intestine are the result of the action of bacteria upon the protein material present. This bacterial action which is the combined effort of many forms of micro-organisms is confined almost exclusively to the large intestine. Some of the products of the putrefaction of proteins are identical with those formed in tryptic digestion, although the decomposition of the protein material is much more extensive when subjected to putrefaction. Some of the more important of the putrefaction products are the following: *Indole*, *skatole*, *paracresol*, *phenol*, *para-oxyphenylpropionic acid*, *para-oxyphenylacetic acid*, *volatile fatty acids*, *hydrogen sulphide*, *methane*, *methyl mercaptan*, *hydrogen*, and *carbon dioxide*, beside *proteoses*, *peptones*, *ammonia*, and *amino acids*. Of these the indole, skatole, phenol, and paracresol appear in part in the urine as ethereal sulphuric<sup>r</sup> acids, whereas the oxyacids mentioned pass unchanged into the urine. The potassium indoxyl sulphate (page 298) content of the urine is a rough indicator of the extent of the putrefaction within the intestine.

The portion of the indole which is excreted in the urine is first subjected to a series of changes within the organism and is subsequently eliminated as *indican*. These changes may be represented thus:

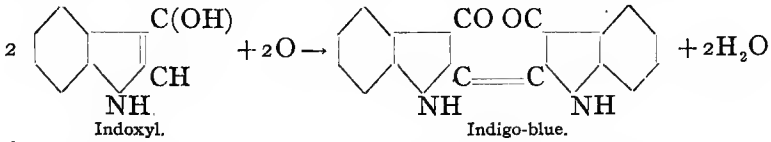


In the presence of potassium salts the indoxyl sulphuric acid is then transformed into indoxyl potassium sulphate (or indican),



and eliminated as such in the urine.

Indican may be decomposed by treatment with concentrated hydrochloric acid (see tests on page 208) into sulphuric acid and indoxyl. The latter body may then be oxidized to form indigo-blue thus:



This same reaction may also occur under pathological conditions *within the organism*, thus giving rise to the appearance of crystals of indigo-blue in the urine.

Skatole is likewise changed within the organism and eliminated in the form of a chromogenic substance. Skatole is, however, of less importance as a putrefaction product than indole and ordinarily occurs in much smaller amount. The tryptophane group of the protein molecule yields the indole and skatole formed in intestinal putrefaction, but the reasons for the transformation of the major portion of this tryptophane into indole and the minor portion into skatole are not well understood. Indole is more toxic than skatole.

Phenol occurs in fairly large amount in certain abnormal conditions of the organism, but ordinarily the amount is very small. It is probably derived from the tyrosine group of the protein molecule. Phenol is conjugated in the liver to form phenyl potassium sulphate and appears in the urine in this form (Baumann and Herter). Para-cresol occurs in the urine as cresyl potassium sulphate.

Regarding the claim of Nencki that methyl mercaptan is formed as a gas during intestinal putrefaction it is an important fact that Herter<sup>1</sup> has been unable to detect the mercaptan in *fresh* feces. He is, therefore, not inclined to accept the theory that methyl mercaptan is formed in *ordinary* intestinal putrefaction but believes that it may be formed in *exceptional cases*. Hydrogen sulphide is, however, formed in all cases of intestinal putrefaction.

It has been shown by Kutscher and his associates<sup>2</sup> that many acids and bases formed in putrefaction and which have been considered as originating alone from bacterial action, may also be formed in certain phases of metabolism in both the plant and animal kingdom. These transformation products of amino acids have been termed "aporrhemas." The following aporrhemas may result from putrefaction processes:

<sup>1</sup> Herter: *Bacterial Infections of the Digestive Tract*, p. 227.

<sup>2</sup> Ackermann and Kutscher; *Zeit. physiol. Chem.*, 69, 265, 1910.

Ackermann: *Ibid.*, 273.

Engelard and Kutscher; *Ibid.*, 282.



Aporrhagma.	Amino Acid Source.
Iminazoletethylamine.....	} Histidine.
Iminazolpropionic acid.....	
Ornithine.....	} Arginine.
Tetramethylendiamine.....	
Aminovalerianic acid.....	Lysine.
Pentamethylendiamine.....	Glutamic acid.
Aminobutyric acid.....	} Aspartic acid.
Alanine.....	
Succinic acid.....	Leucine.
Isovalerianic acid.....	} Phenylalanine.
Phenylethylamine.....	
Phenylacetic acid.....	
Phenylpropionic acid.....	} Tyrosine.
<i>p</i> -Oxyphenylacetic acid.....	
<i>p</i> -Oxyphenylpropionic acid.....	} Tryptophane.
Indole.....	
Skatole.....	
Indolacetic acid.....	
Indolpropionic acid.....	

EXPERIMENTS ON PUTREFACTION PRODUCTS.

In many courses in physiological chemistry the instructors are so limited for time that no extended study of the products of putrefaction can very well be attempted. Under such conditions the scheme here submitted may be used profitably in the way of demonstration. Where the number of students is not too great, a single large putrefaction may be started, and, after the initial distillation, both the resulting distillate and residue may be distributed to the members of the class for individual manipulation.

**Preparation of Putrefaction Mixture.**—Place a weighed mixture of coagulated egg albumin and ground lean meat in a flask or bottle and add approximately 2 liters of water for every kilogram of protein used. Sterilize the vessel and contents, inoculate with the *colon bacillus*, and keep at 40° C. for two or three weeks. If cultures of the colon bacillus are not available, add 60 c.c. of a cold saturated solution of sodium carbonate for every liter of water previously added and inoculate with some putrescent material (pancreas or feces).<sup>1</sup> Mix the putrefaction mixture very thoroughly by shaking and insert a cork furnished with a glass tube to which is attached a wash bottle containing a 3 per cent solution of mercuric cyanide.<sup>2</sup> This device is for the purpose of collecting the methyl mercaptan, a gas formed during the process of putrefaction. It also serves to diminish the odor arising from the putrefying material. Place the putrefaction mixture at 40° C. for two or three weeks and at

<sup>1</sup> Putrefying protein may be prepared by treating 10 grams of finely ground lean meat with 100 c.c. of water and 2 c.c. of a saturated solution of sodium carbonate and keeping the mixture at 40° C. for twenty-four hours.

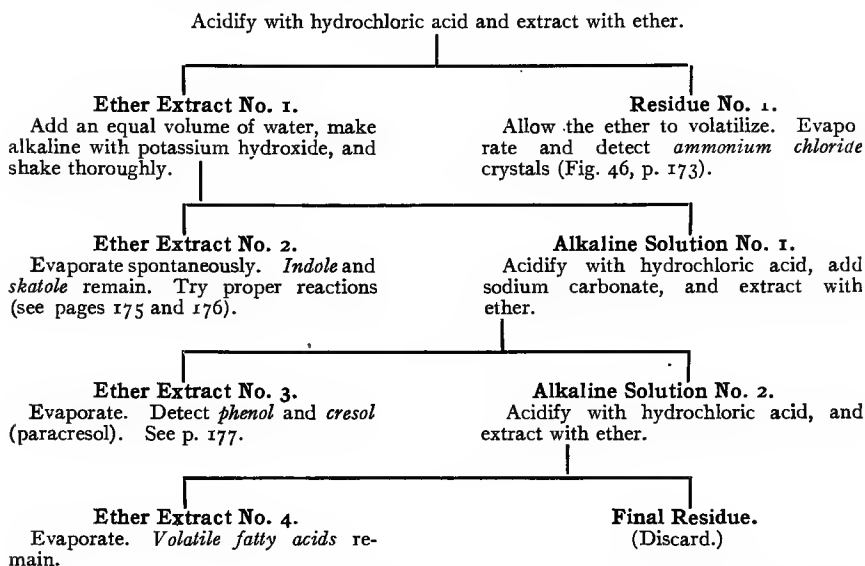
<sup>2</sup> Concentrated sulphuric acid containing a small amount of *isatin* may be used as a substitute for mercuric cyanide. When this modification is employed it is necessary to use calcium chloride tubes to exclude moisture from the isatin solution.

the end of that time make a separation of the products of putrefaction according to the following directions:

Subject the mixture to distillation until the distillate and residue are approximately equal in volume.

## PART I.

### MANIPULATION OF THE DISTILLATE.



### DETAILED DIRECTIONS FOR MAKING THE SEPARATIONS INDICATED IN THE SCHEME.

*Preliminary Ether Extraction.*—This extraction may be conveniently conducted in a separatory funnel. Mix the fluids for extraction in the ratio of *two* volumes of ether to *three* volumes of the distillate. Shake very thoroughly for a few moments, then draw off the extracted fluid and add a new portion of the distillate. Repeat the process until the entire distillate has been extracted. Add a small amount of fresh ether at each extraction to replace that dissolved by the water in the preceding extraction.

*Residue No. 1.*—Unite the portions of the distillate extracted as above and allow the ether to volatilize spontaneously. Evaporate until crystallization begins. Examine the crystals under the microscope. Ammonium chloride predominates. Explain its presence.

*Ether Extract No. 1.*—Add an equal volume of water, render the mixture alkaline with potassium hydroxide, and shake thoroughly by

means of a separatory funnel as before. The *volatile fatty acids*, contained among the putrefaction products, would be dissolved by the alkaline solution (No. 1) whereas any indole or skatole would remain in the ethereal solution (No. 2).

*Alkaline Solution No. 1.*—Acidify with hydrochloric acid and add sodium carbonate solution until the fluid is neutral or slightly acid from the presence of carbonic acid. At this point a portion of the solution, after being heated for a few moments, should possess an alkaline reaction on cooling. Extract the whole mixture with ether in the usual way, using care in the manipulation of the stop cock to

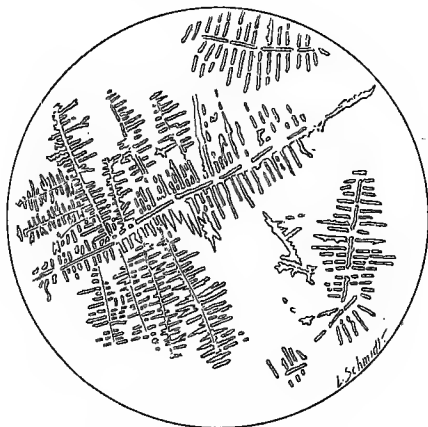


FIG. 46.—AMMONIUM CHLORIDE.

relieve the pressure due to the evolution of carbon dioxide. The ether (Ether Extract No. 3) removes any *phenol* or *cresol* which may be present while the volatile fatty acids will remain in the alkaline solution (No. 2) as alkali salts.

*Ether Extract No. 2.*—Drive off the major portion of the ether at a low temperature on a water-bath and allow the residue to evaporate spontaneously. Indole and skatole should be present here. Prove the presence of these bodies. For tests for indole and skatole see pp. 175 and 176.

*Alkaline Solution No. 2.*—Make strongly acid with hydrochloric acid and extract with a small amount of ether, using a separatory funnel. As carbon dioxide is liberated here, care must be used in the manipulation of the stop cock of the funnel in relieving the pressure within the vessel. The volatile fatty acids are dissolved by the ether (Ether Extract No. 4).

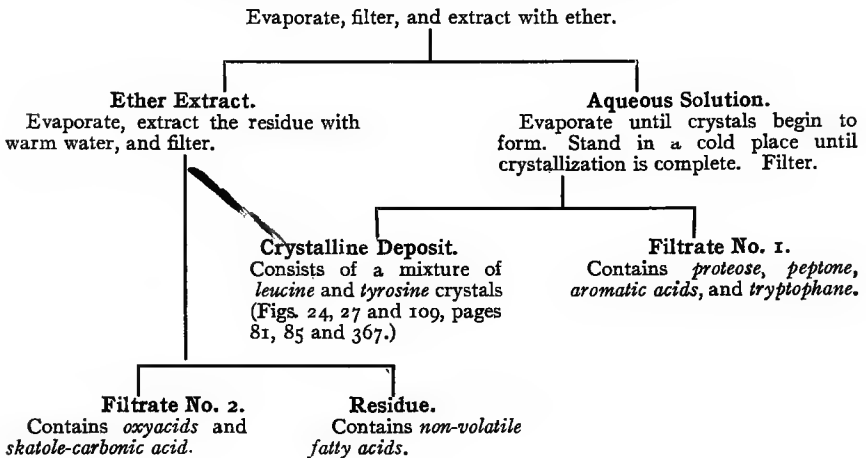
*Ether Extract No. 3.*—Evaporate this ethereal solution on a water-bath. The oily residue contains phenol and cresol. The cresol is

present for the most part as paracresol. Add some water to the oily residue and heat it in a flask. Cool and prove the presence of phenol and cresol. For tests for these bodies see page 177.

*Ether Extract No. 4.*—Evaporate on a water-bath. The volatile fatty acids remain in the residue.

## PART II.

### MANIPULATION OF THE RESIDUE.



### DETAILED DIRECTIONS FOR MAKING THE SEPARATIONS INDICATED IN THE SCHEME.

*Preliminary Ether Extraction.*—This extraction may be conducted in a separatory funnel. In order to make a satisfactory extraction the mixture should be shaken very thoroughly. Separate the ethereal solution from the aqueous portion and treat them according to the directions given on p. 172.

*Ether Extract.*—Evaporate this solution on a *safety* water-bath until the ether has been entirely removed. Extract the residue with warm water and filter.

*Aqueous Solution.*—Evaporate this solution until crystallization begins. Stand the solution in a cold place until no more crystals form. This crystalline mass consists of impure *leucine* and *tyrosine*. Filter off the crystals.

*Crystalline Deposit.*—Examine the crystals under the microscope and compare them with those reproduced in Figs. 24, 27, and 109, pages

81, 85 and 367. Do the forms of the crystals of leucine and tyrosine resemble those previously examined? Make a separation of the leucine and tyrosine and apply typical tests according to directions given on pages 90 and 91.

*Filtrate No. 1.*—Make a test for tryptophane with bromine water (see page 153), and also with the Hopkins-Cole reagent (see page 98). Use the remainder of the filtrate for the separation of proteoses and peptones. Make the separation according to the directions given on page 120.

*Filtrate No. 2.*—This solution contains para-oxyphenylacetic acid, para-oxyphenylpropionic acid and skatole-carbonic acid. Prove the presence of these bodies by appropriate tests. Tests for oxyacids and skatole-carbonic acid are given on page 177.

## TESTS FOR VARIOUS PUTREFACTION PRODUCTS.

### Tests for Indole.

1. **Herter's  $\beta$ -Naphthaquinone Reaction.**—(a) To a dilute aqueous solution of indole (1:500,000) add one drop of a 2 per cent solution of  $\beta$ -naphthaquinone-sodium-monosulphonate. No reaction occurs. Add a drop of a 10 per cent solution of potassium hydroxide and note the gradual development of a blue or blue-green color which fades to green if an excess of the alkali is added. Render the green or blue-green solution acid and note the appearance of a pink color. Heat facilitates the development of the color reaction.

One part of indole in *one million parts* of water may be detected by means of this test if carefully performed.

(b) If the alkali be added to a more concentrated indole solution *before* the introduction of the naphthaquinone the course of the reaction is different, particularly if the indole solution is somewhat more concentrated than that mentioned above and if heat is used. Under these conditions the blue indole compound ultimately forms as fine acicular crystals which rise to the surface.

If we do not wait for the production of the crystalline body but as soon as the blue color forms, shake the aqueous solution with chloroform, the blue color disappears from the solution and the chloroform assumes a *pinkish-red hue*. This is a distinguishing feature of the indole reaction and facilitates the differentiation of indole from other bodies which yield a similar blue color. A very satisfactory method for the quantitative determination of indole is based upon the principle underlying this test.

2. **Konto's Reaction.**—Distil the solution to be tested until only one-third of the original solution remains. Make the distillate alkaline with sodium hydroxide and distil again in order to separate the indole from the phenol, the latter remaining in the residue. Inasmuch as this second distillate generally contains a large amount of ammonia it should be acidified with dilute sulphuric acid and again distilled. To 1 c.c. of this ammonia-free distillate in a test-tube add 3 drops of a 40 per cent solution of formaldehyde and 1 c.c. of concentrated sulphuric acid. Now agitate the mixture and note the appearance of a violet red color if a trace of indole is present. The test is said to serve for the detection of indole when present in a dilution of 1:700,000.

Skatole gives a yellow or brown color under the above conditions.

3. **Cholera-red Reaction.**—To a little of the residue in a test-tube add one-tenth its volume of a 0.02 per cent solution of potassium nitrite and mix thoroughly. Carefully run concentrated sulphuric acid down the side of the tube so that it forms a layer at the bottom. Note the purple color. Neutralize with potassium hydroxide and observe the production of a bluish-green color.

4. **Legal's Reaction.**—To a small amount of the residue in a test-tube add a few drops of a freshly prepared solution of sodium nitroprusside,  $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO} + 2\text{H}_2\text{O}$ . Render alkaline with potassium hydroxide and note the production of a violet color. If the solution is now acidified with glacial acetic acid the violet is transformed into a blue.

5. **Pine Wood Test.**—Moisten a pine splinter with concentrated hydrochloric acid and insert it into the residue. The wood assumes a cherry-red color.

6. **Nitroso-indole Nitrate Test.**—Acidify some of the residue with nitric acid, add a few drops of a potassium nitrite solution and note the production of a red precipitate of nitroso-indole nitrate. If the residue contains but little indole simply a red coloration will result. Compare this result with the result of the similar test on skatole.

### Tests for Skatole.

1. **Herter's Para-dimethylaminobenzaldehyde Reaction.**<sup>1</sup>—To 5 c.c. of the distillate or aqueous solution under examination add 1 c.c. of an acid solution of para-dimethylaminobenzaldehyde<sup>2</sup> and heat the mixture to boiling. A purplish-blue coloration is produced<sup>3</sup>

<sup>1</sup> Herter: *Bacterial Infections of the Digestive Tract*, 1907, p. 141.

<sup>2</sup> Made by dissolving 5 grams of para-dimethylaminobenzaldehyde in 100 c.c. of 10 per cent sulphuric acid.

<sup>3</sup> If the color does not appear add more of the aldehyde solution.

which may be intensified through the addition of a few drops of concentrated hydrochloric acid. If the solution be cooled under running water it loses its purplish tinge of color and becomes a definite blue. The solution at this point may be somewhat opalescent through the separation of uncombined para-dimethylaminobenzaldehyde. Care should be taken not to add an excess of hydrochloric acid inasmuch as the end-reaction has a tendency to fade under the influence of a high acidity.

A rough idea regarding the actual quantity of skatole in a mixture may be obtained by extracting this blue solution with chloroform and subsequently comparing this chloroform solution, by means of a colorimeter (Duboscq), with the maximal reaction, obtained with a skatole solution of known strength.

2. **Color Reaction with Hydrochloric Acid.**—Acidify some of the residue with concentrated hydrochloric acid. Note the production of a violet color.

3. Acidify some of the residue with nitric acid and add a few drops of a potassium nitrite solution. Note the white turbidity. Compare this result with the result of the similar test on indole.

### Tests for Phenol and Cresol.

1. **Color Test.**—Test a little of the solution with Millon's reagent. A red color results. Compare this test with the similar one under Tyrosine (see page 91).

2. **Ferric Chloride Test.**—Add a few drops of *neutral* ferric chloride solution to a little of the residual fluid. A dirty bluish-gray color is formed.

3. **Formation of Bromine Compounds.**—Add some bromine water to a little of the fluid under examination. Note the crystalline precipitate of tribromphenol and tribromcresol.

### Tests for Oxyacids.

1. **Color Test.**—Test a little of the solution with Millon's reagent. A red color results.

2. **Bromine Water Test.**—Add a few drops of bromine water to some of the filtrate. A turbidity or precipitate is observed.

### Test for Skatole-carbonic Acid.

**Ferric Chloride Test.**—Acidify some of the filtrate with hydrochloric acid, add a few drops of ferric chloride solution, and heat. Compare the end-reaction with that given by phenol.

## CHAPTER XI.

### FECES.

The feces is the residual mass of material remaining in the intestine after the full and complete exercise of the digestive and absorptive functions and is ultimately expelled from the body through the rectum. The amount of this fecal discharge varies with the individual and the diet. Upon an ordinary mixed diet various authorities claim that the daily excretion by an adult male will aggregate 110–170 grams with a solid content ranging between 25 and 45 grams; the fecal discharge of such an individual upon a vegetable diet will be much greater and may

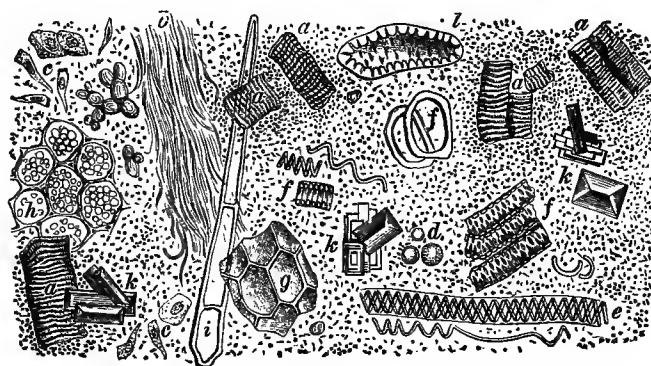


FIG. 47.—MICROSCOPICAL CONSTITUENTS OF FECES. (*v. Jaksch.*)

*a*, Muscle fibers; *b*, connective tissue; *c*, epithelium; *d*, leucocytes; *e*, spiral cells; *f*, *g*, *h*, *i*, various vegetable cells; *k*, "triple phosphate" crystals; *l*, woody vegetable cells; the whole interspersed with innumerable micro-organisms of various kinds.

even be as great as 350 grams and possess a solid content of 75 grams. In the author's own experience the average daily output of moist feces, calculated on the basis of data secured from the examination of over 1,000 stools, was about 100 grams. The variation in the normal daily output being so great renders this factor of very little value for diagnostic purposes, except where the composition of the diet is accurately known. Lesions of the digestive tract, a defective absorptive function, or increased peristalsis as well as an admixture of mucus, pus, blood, and pathological products of the intestinal wall may cause the total amount of excrement to be markedly increased.

The fecal pigment of the normal adult is hydrobilirubin. This



pigment originates from the bilirubin which is secreted into the intestine in the bile, the transformation from bilirubin to hydrobilirubin being brought about through the activity of certain bacteria. Hydrobilirubin is sometimes called stercobilin and bears a close resemblance to urobilin or may even be identical with that pigment. Neither bilirubin nor biliverdin occurs normally in the fecal discharge of adults, although the former may be detected in the excrement of nursing infants. The most important factor, however, in determining the color of the fecal discharge is the diet. A mixed diet, for instance, produces stools which vary in color from light to dark brown, an exclusive meat diet gives rise to a brownish-black stool, whereas the stool resulting from a milk diet is invariably light colored. Certain pigmented foods such as the chlorophyllic vegetables, and various varieties of berries, each afford stools having a characteristic color. Certain drugs act in a similar way to color the fecal discharge. This is well illustrated by the occurrence of green stools following the use of calomel and of black stools after bismuth ingestion. The green color of the calomel stool is generally believed to be due to biliverdin. v. Jaksch, however, claims to have proven this view to be incorrect since he was able to detect hydrobilirubin (or urobilin) but *no biliverdin* in stools after the administration of calomel. The bismuth stool derives its color from the black sulphide which is formed from the subnitrate of bismuth. In cases of biliary obstruction the grayish-white *acholic* stool is formed.



FIG. 48.—HEMATOIDIN CRYSTALS FROM ACHOLIC STOOLS. (v. Jaksch.)

Color of crystals same as the color of those in Fig. 42, p. 161.

Under normal conditions the odor of feces is due to skatole and indole, two bodies formed in the course of putrefactive processes occurring within the intestine (see page 169). Such bodies as methane, methyl mercaptan, and hydrogen sulphide may also add to the disagreeable character of the odor. The intensity of the odor depends to a large degree upon the character of the diet, being very marked in stools from a meat diet, much less marked in stools from a vegetable diet, and frequently hardly detectable in stools from a milk diet. Thus the stool of the infant is ordinarily nearly odorless and any decided odor may generally be readily traced to some pathological source.

A neutral reaction ordinarily predominates in normal stools although slightly alkaline or even acid stools are met with. The acid reaction is encountered much less frequently than the alkaline and then commonly only following a vegetable diet.

Recent experiments<sup>1</sup> in which the actual hydrogen ion concentration of the feces was determined indicated that the reaction of the excreta was uniformly *slightly alkaline*. Pronounced dietary changes *e. g.*, low protein diet, high protein diet, fasting, water drinking with meals, produced at most only minor changes in the reaction of the feces.

The form and consistency of the stool is dependent, in large measure, upon the nature of the diet and particularly upon the quantity of water ingested. Under normal conditions the consistency may vary from a thin, pasty discharge to a firmly formed stool. Stools which are exceedingly thin and watery ordinarily have a pathological significance. In general the feces of the carnivorous animals is of a firmer consistency than that of the herbivora.

The continued ingestion of a diet which is very thoroughly digested and absorbed is frequently accompanied by the formation of dry, hard fecal masses (*scybala*). Constipation generally results, due to the small bulk of the feces and its lack of moisture. To counteract this tendency toward constipation the ingestion of *agar-agar*<sup>2</sup> has been suggested.<sup>3</sup> This agar is relatively indigestible and readily absorbs water thus forming a bulky fecal mass which is sufficiently soft to permit of easy evacuation. The function of agar is not limited to its use in connection with constipation; it may serve in other capacities as an aid to intestinal therapeutics.<sup>4</sup>

It is frequently desirable for clinical or experimental purposes to make an examination of the fecal output which constitutes the residual mass from a certain definite diet. Under such conditions, it is customary to cause the person under observation to ingest some substance, at the beginning and end of the period in question, which shall sufficiently differ in color and consistency from the surrounding feces as to render comparatively easy the differentiation of the feces of that period from the feces of the immediately preceding and succeeding periods. One of the most satisfactory methods of making this "separation" is by means of the ingestion of a gelatin capsule containing about 0.2 gram of powdered charcoal at the beginning and end of the period under observation. This procedure causes the appearance of *two black zones* of charcoal in the fecal mass and thus renders comparatively simple the differentiation of the feces of the intermediate period. Carmine (0.3 gram) may be used in a similar manner and forms two *dark red zones*. Some similar method for the "separation of feces" is universally practised in connection with the scientifically accurate type of nutrition or metabolism experiment

<sup>1</sup> Howe and Hawk; *Jour. Biol. Chem.*, 11, 129, 1912.

<sup>2</sup> Agar-agar is a product prepared from certain types of Asiatic sea-weed. It is a carbohydrate and is classified as a *galactan* in the polysaccharide group.

<sup>3</sup> Mendel: *Zent. f. ges. Physiol. u. Path. des Stoffw.*, No. 17, p. 1, 1908; Schmidt: *Münch. med. Woch.*, 52, 1970, 1905.

<sup>4</sup> Einhorn: *Berl. klin. Woch.*, 49, 113, 1912.

which embraces the collection of useful data regarding the income and outgo of nitrogen, and other elements.

Among the macroscopical constituents of the feces may be mentioned the following: Intestinal parasites, undigested food particles, gall stones, pathological products of the intestinal wall, enteroliths, intestinal sand, and objects which have been accidentally swallowed.

The fecal constituents which at various times and under different conditions may be detected by the use of the microscope are as follows: Constituents derived from the food, such as *muscles fibers*, *connective-tissue shreds*, *starch granules*, and *fat*; formed elements derived from the intestinal tract, such as *epithelium*, *erythrocytes*, and *leucocytes*; *mucus*; *pus corpuscles*; *parasites* and *bacteria*. In addition to the constituents named the following *crystalline deposits* may be detected: *cholesterol*, *soaps*, *fatty acid*, *fat*, *bismuth sulphide*, *hæmatoidin*, "*triple phosphate*," *Charcot-Leyden crystals*, and the *oxalate*, *carbonate*, *phosphate*, *sulphate*, and *lactate* of calcium.



FIG. 49.—CHARCOT-LEYDEN CRYSTALS.

The detection of minute quantities of blood in the feces ("occult blood") has recently become a recognized aid to a correct diagnosis of certain disorders.

In these instances the hemorrhage is ordinarily so slight that the identification by means of macroscopical characteristics as well as the microscopical identification through the detection of erythrocytes are both unsatisfactory in their results. Of the tests given for the detection of "occult blood" the *benzidine reaction* and the *phenolphthalein* and *aloin-turpentine tests* (page 185) are probably the most satisfactory. Since "occult blood" occurs with considerable regularity and frequency in gastrointestinal cancer and in gastric and duodenal ulcer, its detection in the feces is of especial value as an aid to a correct diagnosis of these disorders.

It has been quite clearly shown that the intestine of the newly born is sterile. However, this condition is quickly altered and bacteria may be present in the feces before or after the first ingestion of food. There are three possible means of infecting the intestine, *i. e.*, by way of the mouth or anus or through the blood. The infection by means of the blood seldom occurs except under pathological conditions, thus limiting the general infection to the mouth and anus.

In infants with pronounced constipation two-thirds of the dry substance of the stools has been found to consist of bacteria. In the stools of normal adults probably about one-third of the dry substance is bacteria.<sup>1</sup>

<sup>1</sup> Schittenhelm and Tollens found bacteria to comprise 42 per cent of the dry matter. This value is, however, undoubtedly too high.

The average excretion of dry bacteria in twenty-four hours for an adult is about 8 grams. The output of fecal bacteria has been found to undergo a decrease under the influence of water drinking with meals.<sup>1</sup> There was also a decrease in intestinal putrefaction,<sup>2</sup> a fact which indicates that at least a part of the bacterial deficit was made up of putrefactive organisms. Over 50 per cent of the total nitrogen of feces has been shown to be *bacterial nitrogen*.<sup>3</sup>

Various enzymes have been detected in the feces. The first one so demonstrated was pancreatic amylase.<sup>4</sup> The amylase content of the feces is believed to be an index of the activity of the pancreatic function.<sup>5</sup> The excretion of this enzyme has been found to increase under the influence of water drinking with meals.<sup>6</sup> Other enzymes which have been found in the feces under various conditions are trypsin, rennin, maltase, sucrase, lactase, nuclease and lipase.<sup>7</sup>

Some of the more important organisms met with in the feces are the following:<sup>8</sup> *B. coli*, *B. lactis aërogenes*, *Bact. Welchii*, *B. bifidus*, and *coccal forms*. Of these the first three types mentioned are *gas-forming* organisms. The production of gas by the fecal flora in dextrose-bouillon is subject to great variations under pathological conditions: alterations in the diet of normal persons will also cause wide fluctuations. In this connection Herter has observed a marked reduction or even complete cessation of gas production by the mixed fecal bacteria while considerable doses of benzoate were being given. A return to the former plane of gas production followed the discontinuation of the benzoate.<sup>9</sup> Data as to the production of gas are of considerable importance in a diagnostic way although the exact cause of the variations is not yet established. It should be borne in mind in this connection that gas volumes are frequently variable with the same individual. For this reason it is necessary in every instance to follow the gas production for a considerable period of time before drawing conclusions.<sup>10</sup>

The nitrogen present in the feces consists principally of *bacteria*, *unabsorbed intestinal secretions*, *epithelial cells*, *mucus material* and *food residues*. In the early days of nutrition study the fecal nitrogen was

<sup>1</sup> Mattill and Hawk: *Jour. Am. Chem. Soc.*, 33, 1999, 1911; Blatherwick, Sherwin and Hawk: *Jour. Biol. Chem.*, 11, viii, 1912 (Proceedings).

<sup>2</sup> Hattrem and Hawk: *Arch. Int. Med.*, 7, 610, 1911; Blatherwick, Sherwin and Hawk: *loc. cit.*

<sup>3</sup> MacNeal, Latzer and Kerr: *Jour. Inf. Dis.*, 6, 123, 1909; Mattill and Hawk: *Jour. Exp. Med.*, 14, 433, 1911; Blatherwick and Hawk: Unpublished data.

<sup>4</sup> Wegscheider: *Inaug. Diss.*, Strassburg, 1875.

<sup>5</sup> Wohlgenuth: *Berl. klin. Woch.*, 47, 3, 92, 1910.

<sup>6</sup> Hawk: *Arch. Int. Med.*, 8, 382, 1911.

<sup>7</sup> Ury: *Biochem. Zeit.*, 23, 152, 1909.

<sup>8</sup> Herter and Kendall: *Journal of Biological Chemistry*, 5, 283, 1908.

<sup>9</sup> Private communication from Professor C. A. Herter.

<sup>10</sup> Herter and Kendall: *loc. cit.*

believed to consist principally of food residues. We now know that such residues ordinarily make up but a small part of the nitrogen quota of the stools of normal individuals who exercise normal mastication.<sup>1</sup> When meat has been "bolted," however, from 1/2 gram to 16 grams of macroscopical meat residues has been found in a single stool.<sup>2</sup> The phrase "metabolic product nitrogen" is frequently used as a designation for all fecal nitrogen except that present as food residues and bacteria. Bacteria cannot logically be classed under "metabolic" nitrogen since they doubtless develop at the expense of food nitrogen as well as at the expense of that in the form of intestinal secretions. In the accurate study of "protein utilization"<sup>3</sup> a correction should be made for "metabolic nitrogen." Data regarding the output of metabolic nitrogen may be secured by determining the fecal nitrogen excretion on a diet of proper energy value but *containing no nitrogen*.<sup>4</sup> Agar-agar may be utilized advantageously in connection with such a nitrogen-free diet.

Feces is still excreted from the intestine even when no food is ingested. Carefully conducted fasting experiments have demonstrated this. A dog nourished on an ordinary diet to which *bone ash* has been added will excrete a *grey feces*. When fasted such an animal will, after a few days, excrete a small amount of a greenish-brown mass, *containing no bone ash*. This is *fasting feces*. It is of a pitch-like consistency and turns black on contact with the air.<sup>5</sup> Adult fasting men have been found to excrete 7-8 grams of feces per day, the daily nitrogen value being about 0.1 gram.<sup>6</sup> No separating medium such as charcoal or carmine (p. 180) should be used in differentiating fasting feces.

In recent years the examination of feces for evidences of parasitism (detection of parasites and their ova) has taken on an added importance. The investigation of the hookworm has been particularly developed. (For methods and discussion see *Bulletin* 135, Bureau of Animal Industry, U. S. Department of Agriculture, 1911 (M. C. Hall.)

For diagnostic purposes the macroscopical and microscopical examinations of the feces ordinarily yield much more satisfactory data than are secured from its chemical examination.

<sup>1</sup> Kermauner: *Zeit. für Biol.*, 35, 316, 1897.

<sup>2</sup> Foster and Hawk: *Proceedings of Eighth International Congress of Applied Chem.*, New York, September, 1912.

<sup>3</sup> The percentage of the ingested protein which is absorbed from the intestine. This may be calculated by subtracting the metabolic nitrogen from the total fecal nitrogen and dividing this value by the food nitrogen.

<sup>4</sup> Tsuboi: *Zeit. für Biol.*, 35, 68, 1897; Mendel and Fine: *Jour. Biol. Chem.*, 11, 5, 1912.

<sup>5</sup> Howe and Hawk: *Jour. Am. Chem. Soc.*, 33, 215, 1911.

<sup>6</sup> Howe, Mattill and Hawk: *Ibid.*, 33, 568, 1911.

## EXPERIMENTS ON FECES.

1. **Macroscopical Examination.**—If the stool is watery pour it into a shallow dish and examine directly. If it is firm or pasty it should be treated with water and carefully stirred before the examination for macroscopical constituents is attempted.

The macroscopical constituents may be collected very satisfactorily by means of a Boas sieve (Fig. 50). This sieve is constructed of two easily detachable hemispheres which are held together by means of a bayonet catch. In using the apparatus the feces is spread out upon a very fine sieve contained in the lower hemisphere and a stream of water is allowed to play upon it through the medium of an opening in the upper hemisphere. The apparatus is provided with an orifice in the upper hemisphere through which the feces may be stirred by means of a glass rod during the washing process. After 15–30 minutes' washing nothing but the coarse fecal constituents remain upon the sieve.

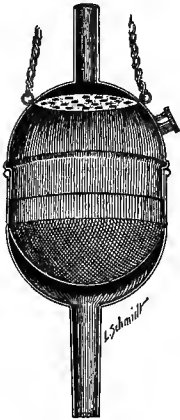


FIG. 50.—BOAS' SIEVE.

2. **Microscopical Examination.**—Watery stools should be placed in a shallow dish, thoroughly mixed, and a small amount removed to a slide for examination. Stools of a firm or pasty consistency should be rubbed up in a mortar with physiological salt solution and a small portion of the resulting mixture transferred to a slide for examination. In normal feces look for *food particles*, *bacteria* and *crystalline bodies*. In pathological stools, in addition to these substances, look for *animal parasites* and *pathological products* of the intestinal wall. See Fig. 47, page 178.

3. **Reaction.**—Thoroughly mix the feces and apply moist red and blue litmus papers to the surface. If the stool is hard it should be mixed with water before the reaction is taken. Examine the stool as soon after defecation as is convenient, since the *reaction* may change very rapidly. The *reaction* of the normal stools of adult man is ordinarily neutral or faintly alkaline to litmus, but seldom acid. Infants' stools are generally acid in reaction. Try the reaction to Congo red paper. Also test the reaction of fecal extract to phenolphthalein.

4. **Starch.**—If any imperfectly cooked starch-containing food has been ingested it will be possible to detect starch granules by a microscopical examination of the feces. If the granules are not detected by a microscopical examination, the feces should be placed in an evaporating dish or casserole and boiled with water for a few minutes. Filter and test the filtrate by the iodine test in the usual way (see page 50).

5. **Cholesterol and Fat.**—Extract the *dry* feces with ether in a Soxhlet apparatus (see Fig. 136). If this apparatus is not available transfer the dry feces to a flask, add ether, and shake frequently for a few hours. Filter and remove the ether by evaporation. The residue contains cholesterol and the mixed fats of the feces. For every gram of fat add about 1 1/2 gram of solid potassium hydroxide and 25 c.c. of 95 per cent alcohol and boil in a flask on a water-bath for one-half hour, maintaining the volume of alcohol constant. This alcoholic-potash has saponified the mixed fats and we now have a mixture of soaps and cholesterol. Add sodium chloride, in substance, to the mixture and extract with ether to dissolve out the cholesterol. Remove the ether by evaporation and examine the residue microscopically for cholesterol crystals. Try any of the other tests for cholesterol as given on page 272.

6. **Blood.**—Undecomposed blood may be detected macroscopically. If uncertain, look for erythrocytes under the microscope, and spectroscopically for the spectrum of oxyhæmoglobin (see Absorption Spectra, Plate I).

In case the blood has been altered or is present in minute amount ("occult blood"), and cannot be detected by the means just mentioned, the following tests may be tried:

(a) *Benzidine Reaction.*—Make a thin fecal suspension using about 5 c.c. of distilled water, and heat it to boiling to render oxidizing enzymes inactive. To 2 c.c. of a saturated solution of benzidine in glacial acetic acid add 3 c.c. of 3 per cent hydrogen peroxide and 2-3 drops of the cooled fecal suspension. A clear *green* or *blue* color appears within 1-2 minutes in the presence of blood. If the mixture is *not shaken* a ring of color will form at the top. Minute traces of blood are more easily detected by the latter procedure.

(b) *Phenolphthalein Test.*<sup>1</sup>—Make a thin fecal suspension using about 5 c.c. of distilled water. Heat to boiling,<sup>2</sup> cool and add 2 c.c. of the suspension to 1 c.c. of the phenolphthalein reagent<sup>3</sup> and a few drops of hydrogen peroxide. A *pink* or *red* color promptly forms in the presence of blood.

(c) *Aloin-turpentine Test.*—Mix the stool very thoroughly and take about 5 grams of the mixture for the test. Reduce this sample to a semi-fluid mass by means of distilled water and extract very thoroughly

<sup>1</sup> Boas: *Deut. Med. Woch.*, 37, 62, 1911.

<sup>2</sup> Boas suggests using an ether extract of the fecal suspension thus eliminating the necessity of boiling. However, oxidizing enzymes are the main sources of error here and the action is easily and effectively eliminated by boiling. (See White: *Boston Medical and Surgical Journal*, 164, 876, 1911.)

<sup>3</sup> Prepared by dissolving 1-2 grams of phenolphthalein and 25 grams of KOH in 100 c.c. of distilled water. Add 10 grams of powdered zinc and heat gently until the solution is decolorized. Prepared in this way the solution will not deteriorate on standing.

with an equal volume of ether to remove any fat which may be present. Now treat the extracted feces with one-third its volume of glacial acetic acid and 10 c.c. of ether and extract very thoroughly as before. The acid-ether extract will rise to the top and may be removed.

Introduce 2-3 c.c. of this acid-ether solution into a test-tube, add an equal volume of a dilute solution of aloin in 70 per cent alcohol and 2-3 c.c. of ozonized turpentine and shake the tube gently. If blood is present the entire volume of fluid ordinarily becomes pink and finally cherry-red. In some instances the color will be limited to the aloin solution which sinks to the bottom. This color reaction should occur within fifteen minutes in order to indicate a positive test for blood, since the aloin will turn red of itself if allowed to stand for a longer period. The color is ordinarily light yellow in a negative test. Hydrogen peroxide is not a satisfactory substitute for turpentine in the test.

(d) *Weber's Guaiac Test*.—Mix a little feces with 30 per cent acetic acid to form a fluid mass. Transfer to a test-tube and extract with ether. If blood is present the ether will assume a brownish-red color. Filter off the ether extract and to a portion of the filtrate add an alcoholic solution of guaiac (strength about 1:60),<sup>1</sup> drop by drop, until the fluid becomes turbid. Now add hydrogen peroxide or old turpentine. In the presence of blood a blue color is produced (see page 209).

(e) *Cowie's Guaiac Test*.—To 1 gram of moist feces add 4-5 c.c. of glacial acetic acid and extract the mixture with 30 c.c. of ether. To 1-2 c.c. of the extract *add an equal volume of water*, agitate the mixture, introduce a few granules of powdered guaiac resin, and after bringing the resin into solution, gradually add 30 drops of old turpentine or hydrogen peroxide. A blue color indicates the presence of blood. Cowie claims that by means of this test an intestinal hemorrhage of 1 gram can easily be detected by an examination of the feces.

(f) *Acid-hæmatin*.—Examine some of the ethereal extract from Experiment (d) spectroscopically. Note the typical spectrum of acid-hæmatin (see Absorption Spectra, Plate II).

7. **Hydrobilirubin.** *Schmidt's Test*.—Rub up a small amount of feces in a mortar with a concentrated aqueous solution of mercuric chloride. Transfer to a shallow, flat-bottomed dish and allow to stand 6-24 hours. The presence of hydrobilirubin will be indicated by a deep red color being imparted to the particles of feces containing this pigment. This red color is due to the formation of hydrobilirubin-mercury. If unaltered bilirubin is present in any portion of the feces that portion will be green in color due to the oxidation of bilirubin to biliverdin.

<sup>1</sup> Buckmaster advises the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiac resin.



Another method for the detection of hydrobilirubin is the following: Treat the dry feces with absolute alcohol acidified with sulphuric acid and shake thoroughly. The acidified alcohol extracts the pigment and assumes a reddish color. Examine a little of this fluid spectroscopically and note the typical spectrum of hydrobilirubin (Absorption Spectra, Plate II).

8. **Bilirubin.**<sup>1</sup> (a) *Gmelin's Test.*—Place a few drops of concentrated nitric acid in an evaporating dish or on a porcelain test-tablet and allow a few drops of the feces and water to mix with it. The usual play of colors of Gmelin's test is produced, *i. e.*, green, blue, violet, red, and yellow. If so desired, this test may be executed on a slide and observed under a microscope.

(b) *Huppert's Test.*—Treat the feces with water to form a semi-fluid mass, add an equal amount of milk of lime, shake thoroughly, and filter. Wash the precipitate with water, then transfer both the paper and the precipitate to a small beaker or flask, add a small amount of 95 per cent alcohol acidified slightly with sulphuric acid, and heat to boiling on a water-bath. The presence of bilirubin is indicated by the alcohol assuming a green color.

Steensma advises the addition of a drop of a 0.5 per cent solution of sodium nitrite to the acid-alcohol mixture before warming on the water-bath. Try this modification also.

9. **Bile Acids.**—Extract a small amount of feces with alcohol and filter. Evaporate the filtrate on a water-bath to drive off the alcohol and dissolve the residue in water made slightly alkaline with potassium hydroxide. Upon this aqueous solution try any of the tests for bile acids given on page 163.

10. **Caseinogen.**—Extract the fresh feces first with a dilute solution of sodium chloride, and later with water acidified with dilute acetic acid, to remove soluble proteins. Now extract the feces with 0.5 per cent sodium carbonate and filter. Add dilute acetic acid to the filtrate to precipitate the caseinogen, being careful not to add an excess of the reagent as the caseinogen would dissolve. Filter off the caseinogen and test it according to directions given on page 241. Caseinogen is found principally in the feces of children who have been fed a milk diet. Mucin would also be extracted by the dilute alkali, if present in the feces. What test could you make on the newly precipitated body to differentiate between mucin and caseinogen?

11. **Nucleoprotein.**—Mix the stool thoroughly with water, transfer to a flask, and add an equal amount of saturated lime water. Shake

<sup>1</sup> The detection of bilirubin in the feces is comparatively simple provided it is not accompanied by other pigments. When other pigments are present, however, it is difficult to detect the bilirubin and, at times, may be found impossible.

frequently for a few hours, filter, and precipitate the nucleo-protein with acetic acid. Filter off this precipitate and test it as follows:

- (a) *Phosphorus*.—Test for phosphorus by fusion (see page 271).
- (b) *Solubility*.—Try the solubility in the ordinary solvents.
- (c) *Protein Color Test*.—Try any of the protein color tests.

What proof have you that the above body was not mucin? What other test can you use to differentiate between nucleoprotein and mucin?

12. **Albumin and Globulin**.—Extract the fresh feces with a dilute solution of sodium chloride. (The preliminary extract from the preparation of caseinogen (10), above, may be utilized here.) Filter, and saturate a portion of the filtrate with sodium chloride in substance. A precipitate signifies globulin. Filter off the precipitate and acidify the filtrate slightly with dilute acetic acid. A precipitate at this point signifies albumin. Make a protein color test on each of these bodies.

13. **Proteose and Peptone**.—Heat to boiling the portion of the sodium chloride extract not used in the last experiment. Filter off the coagulum, if any forms. Acidify the filtrate slightly with acetic acid and saturate with sodium chloride in substance. A precipitate here indicates proteose. Filter it off and test it according to directions given on page 120. Test the filtrate for peptone by the biuret test.

14. **Inorganic Constituents**.—Prepare a dilute aqueous solution of dry feces and decolorize it by means of purified animal charcoal. Make the following tests upon the clear solution.

- (a) *Chlorides*.—Acidify with nitric acid and add silver nitrate.
- (b) *Phosphates*.—Acidify with nitric acid, add molybdic solution, and warm gently.
- (c) *Sulphates*.—Acidify with hydrochloric acid, add barium chloride, and warm.

15. **Konto's Reaction for Indole**.—Rub up the stool with water to form a thin paste. From this point the test is the same as for the detection of indole in putrefaction mixtures (see page 176).

16. **Schmidt's Nuclei Test**.—This test serves as an aid to the diagnosis of pancreatic insufficiency. The test is founded upon the theory that cell nuclei are digestible *only* in pancreatic juice, and therefore that the appearance in the feces of such nuclei indicates insufficiency of pancreatic secretion. The procedure is as follows: Cubes of fresh beef about one-half centimeter square are enclosed in small gauze bags and ingested with a test meal. Subsequently the fecal mass resulting from this test-meal is examined, the bag opened, and the condition of the enclosed residue determined. Under normal conditions the nuclei would be digested. Therefore if the nuclei are found to be for the most part undigested, and the intervening period has been sufficient to permit of

the full activity of the pancreatic function (at least six hours), it may be considered a sign of pancreatic insufficiency.

It has been claimed by Steele that under certain conditions the non-digestion of the nuclei may indicate a general lowering of the digestive power rather than a true pancreatic insufficiency.

Kashiwado<sup>1</sup> has recently suggested the use of stained cell nuclei in this test.

17. **Einhorn's Bead Test.**<sup>2</sup>—This is a method for testing the digestive function. In some respects it is similar to Sahli's desmoid reaction. The procedure consists in wrapping the material under examination (catgut, fish-bone, raw beef, cooked potatoes, thymus gland or mutton fat, etc.) in gauze to which glass beads of various colors are attached and enclosing gauze and beads in a gelatine capsule.<sup>3</sup> The gelatine capsule is swallowed and the beads serve to facilitate the separation of the gauze from the feces. The residue within the gauze is then examined. If beads appear in much less than 24 hours an accelerated motility is indicated, whereas an interval of 48 hours or over elapsing indicates retarded motility. If gastric function alone is to be studied silk threads are attached to the beads and the latter are withdrawn and examined before they have passed into the intestine.

18. **"Separation" of Feces.**—In order to become familiar with the method ordinarily utilized in metabolism experiments to differentiate the feces which corresponds to the food ingested during any given interval, and at the same time to secure data as to the length of time necessary for ingested substances to pass through the alimentary tract proceed as follows: Just before one of the three meals of the day ingest a gelatine capsule (No. 00) containing 0.2–0.3 of a gram of carmine or charcoal. Make an inspection of all stools subsequently dropped and note the time interval elapsing between the ingestion of the capsule and the appearance of its contents in the feces. Under normal conditions this period is ordinarily 24 hours.

19. **Quantitative Determination of Fecal Amylase (The Author's<sup>4</sup> Modification of Wohlgemuth's<sup>5</sup> Method).**—Weigh accurately about 2 grams of fresh feces into a mortar,<sup>6</sup> add 8 c.c. of a phosphate-chloride solution (0.1 mol dihydrogen sodium phosphate and 0.2 mol disodium hydrogen phosphate per liter of 1 per cent sodium chloride), 2 c.c. at a time, rubbing the feces mixture to a homogeneous consistency after each ad-

<sup>1</sup> Kashiwado: *Deut. Arch. Klin. Med.*, 104, 584, 1911.

<sup>2</sup> Einhorn: *The Post-Graduate*, May 1912; *Boas' Arch.*, 12, 26, 1906; 13, 35, 1907; *Ibid.*, 475; 15, part 2, 1909.

<sup>3</sup> Ordinarily two substances are attached to each bead, three beads tied together and enclosed in one capsule. Test capsules may be obtained from Eimer and Amend, New York.

<sup>4</sup> Hawk: *Arch. Int. Med.*, 8, 552, 1911.

<sup>5</sup> Wohlgemuth: *Berl. klin. Woch.*, 47, 3, 92, 1910; also see chapter on Enzymes, this book.

<sup>6</sup> Duplicate determinations should be made.

dition of the extraction medium. Permit the mixture to stand at room temperature for a half-hour with frequent stirring. We now have a neutral fecal suspension. Transfer this suspension to a 15 c.c. graduated centrifuge tube, being sure to wash the mortar and pestle carefully with the phosphate-chloride solution and add all washings to the suspension in the centrifuge tube. The suspension is now made up to the 15 c.c. mark with the phosphate-chloride solution and centrifugated for a fifteen-minute period, or longer if necessary, to secure satisfactory sedimentation. At this point, read and record the height of the sediment column. Remove the supernatant liquid by means of a bent pipette, transfer it to a 50 c.c. volumetric flask and dilute it to the 50 c.c. mark with the phosphate-chloride solution. Mix the fecal extract thoroughly by shaking and determine its amylolytic activity. For this purpose a series of six graduated tubes is prepared, containing volumes of the extract ranging from 2.5 c.c. to 0.078 c.c. Each of the intermediate tubes in this series will thus contain one-half as much fluid as the preceding tube. Now make the contents of each tube 2.5 c.c. by means of the phosphate-chloride solution in order to secure a uniform electrolyte concentration. Introduce 5 c.c. of a 1 per cent soluble starch solution<sup>1</sup> and three drops of toluol into each tube, thoroughly mix the contents by shaking, close the tubes by means of stoppers and place them in an incubator at 38° C. for twenty-four hours. At the end of this time remove the tubes, fill each to within half an inch of the top with ice-water, add one drop of tenth-normal iodine solution, thoroughly mix the contents and examine the tubes carefully with the aid of a strong light. Select the last tube in the series which shows entire absence of blue color, thus indicating that the starch has been completely transformed into dextrin and sugar, and calculate the amylolytic activity on the basis of this dilution. In case of indecision between two tubes, add an extra drop of the iodine solution and observe them again.<sup>2</sup>

<sup>1</sup> In preparing the 1 per cent solution, the weighed starch powder should be dissolved in cold distilled water in a casserole and stirred until a homogeneous suspension is obtained. The mixture should then be heated with constant stirring, until it is clear. This ordinarily takes from eight to ten minutes. A slightly opaque solution is thus obtained, which should be cooled and made up to the proper volume before using.

<sup>2</sup> Theoretically we would expect the colors to range from a light yellow to a dark blue, with red tubes holding an intermediate position in the series. This color sequence does often occur, but its occurrence is far from universal. Many times the first tubes in the series, *i. e.*, those containing the largest quantities of the fecal extract, will exhibit a bluish cast of color which should not be confused with the starch color reaction. When these blue tubes are present, they are generally followed by yellow, red and blue tubes in order, the final blue tube, of course, being the regulation starch reaction. Occasionally greenish colors will be obtained to the left of the red color. It also sometimes happens that it is somewhat difficult to determine in which tube to the right of the red color the starch blue color is first detected, unless the tube be examined carefully before a strong light. In every instance, however, when these blue and green colors are observed, it is noted that tubes possessing the true dextrin red color are always present between these tubes and the tubes possessing the true starch blue color. It is evident, therefore, that these bluish tints in the tubes to the left of the dextrin color cannot be due to the presence of starch. The cause of the blue color reaction in the first tubes of the series has not been ascertained as yet.

The amyolytic value, Df, of a given stool, may be expressed in terms of 1 c.c. of the sediment obtained by centrifugation as above described. For example, if it is found that 0.31 c.c. of the phosphate-chloride extract of the stool acting at 38° C. for twenty-four hours completely transformed the starch in 5 c.c. of a 1 per cent starch solution, then we would have the following proportion:

$$0.31 : 5 \text{ (c.c. starch)} :: 1 \text{ (c.c. extract)} : X$$

The value of X in this case is 16.1, which means that 1 c.c. of the fecal extract possesses the power of completely digesting 16.1 c.c. of a 1 per cent starch solution in twenty-four hours at 38° C.

Inasmuch as stools vary so greatly as to water content, it is essential to an accurate comparison of stools that such comparison be made on the basis of the solid matter. Supposing, for example, that in the above determination we had 6.2 c.c. of sediment. Since the supernatant fluid was removed and made up to 50 c.c. before testing its amyolytic value, it is evident that 1 c.c. of this sediment is equivalent to 8.1 c.c. of extract. Therefore, in order to derive the amyolytic value of 1 c.c. of sediment, we must multiply the value (16.1) as obtained above for the extract, by 8.1. This yields 130.4 and enables us to express the activity as follows:

$$\text{Df} \frac{38 \text{ c}}{24 \text{ h}} = 130.4$$

The above method of calculation is that suggested by Wohlgemuth. In case time and facilities permit of the determination of the moisture content of the feces, it is much more accurate and satisfactory to place the amyolytic values of the stools on a "gram of dry matter" basis. The amyolytic values of the stools are expressed as the number of cubic centimeters of 1 per cent starch solution which the amylase content of 1 gram of dry feces is capable of digesting.

**20. Quantitative Determination of Fecal Bacteria.**<sup>1</sup>—The method is a simplification of MacNeal's adaptation of the Strasburger procedure.<sup>2</sup> About two grams of feces are accurately weighed and placed in a 50 c.c. centrifuge tube. To the feces in the tube a few drops of 0.2 per cent hydrochloric acid are added, and the material is mixed to a smooth paste by means of a glass rod. Further amounts of the acid are added with continued crushing and stirring until the material is thoroughly suspended. The tube is then whirled in the centrifuge at high speed for one half to one minute. The suspension is found sedimented into more or less definite layers, the uppermost of which is fairly free from the larger particles. The upper and more liquid portion of the suspension is now drawn off by

<sup>1</sup> Mattill and Hawk: *Jour. Exp. Med.*, 14, 433, 1911.

<sup>2</sup> MacNeal, Latzer and Kerr, *Jour. Inf. Dis.*, 6, 123, 1909.

means of a pipette and transferred to a beaker.<sup>1</sup> The sediment remaining in the tube is again rubbed up with the glass rod with the addition of further amounts of dilute acid, and again centrifugalized for one half to one minute. The supernatant liquid is pipetted off and added to the first, the same pipette being used for the one determination throughout.<sup>2</sup> A third portion of the dilute acid is then added to the sediment, which is again mixed by stirring and again centrifugalized. All the washings are added to the first one, and during the process care is taken to wash the material from the walls and mouth of the centrifuge tube down into it. Finally, when the sediment is sufficiently free from bacteria, the various remaining particles are visibly clean, and the supernatant liquid after centrifugalization remains almost clear. This is removed to the beaker in which are now practically all the bacteria present in the original portion of feces, together with some solid matter not yet separated. In the centrifuge tubes there is a considerable amount of bacteria-free solid matter.

The suspension is now transferred to the same centrifuge tube, centrifugalized for a minute, and the supernatant liquid transferred to a clean beaker by means of the same pipette. The tube is then refilled from the first beaker and thus all the suspension centrifugalized a second time. The beaker is finally carefully washed with the aid of a rubber-tipped glass rod, the second sediment in the centrifuge tube is washed free of bacteria by means of this wash water and by successive portions of the dilute acid, and the supernatant liquid after centrifugalization is added to the contents of the second beaker. The second clean sediment is added to the first. The bacterial suspension now in the second beaker is again centrifugalized in the same way and a third portion of bacteria-free sediment is separated. Frequently a fourth serial centrifugalization is performed—always if the third sediment is of appreciable quantity. At all stages of the separation, small portions of the dilute hydrochloric acid are used, so that the final suspension shall not be too voluminous. Ordinarily it amounts to 125 to 200 c.c. At the same time, the final amount of fluid should not be too small, as shown by Ehrenpfordt,<sup>3</sup> because the viscosity accompanying increased concentration prevents proper and complete sedimentation.

To the final bacterial suspension an equal volume of alcohol is added and the beaker set aside to concentrate. A water bath at 50° to 60° C. is very satisfactory. After two or three days, when the liquid is concentrated to about 50 c.c., the beaker is removed and about 200 c.c. of alcohol

<sup>1</sup> A 25 c.c. pipette is the most satisfactory size; to facilitate observation, the delivery tube is bent near the bulb to an angle of about 120 degrees.

<sup>2</sup> A convenient support for the pipettes is a wire spring on a glass base, such as is used on a desk for pen-holders. The delivery tube, just where it is bent, is inserted between the wires, and any liquid not delivered collects in the bend of the tube.

<sup>3</sup> Ehrenpfordt: *Zeit. exp. Path. Ther.*, 7, 455, 1909.

are added. The beaker is covered and allowed to stand at room temperature for twenty-four hours. At the end of this time the bacterial substance is generally settled, so that most of the clear supernatant liquid, of dark brown color, can be directly siphoned off without loss of solid matter. The remainder is then transferred to centrifuge tubes, centrifugalized, and the remaining clear liquid pipetted off.<sup>1</sup> The sediment consists of the bodies of the bacteria, and is transferred to a Kjeldahl flask for nitrogen determination. This is the bacterial nitrogen. Where a determination of bacterial dry substance is desired, the sediment of bacteria is extracted by absolute alcohol and ether in succession, transferred to a weighed porcelain crucible, and dried at 102° C. to constant weight. This dried sample is then used in the nitrogen determination. Our procedure differs from that of MacNeal in that the bacterial dry matter is not determined. A saving of about seven days' time and of considerable labor is accomplished by this omission.

Inasmuch as it has been shown by various investigators that such bacteria as are present in the feces contain on the average about 11 per cent of nitrogen, the values for bacterial nitrogen as determined by our method may conveniently serve as a basis for the calculation of the actual output of bacterial substance.

<sup>1</sup>In more recent work (see Blatherwick and Hawk: unpublished) it has been found advantageous to centrifugalize with alcohol and ether in succession before transferring the bacterial cells to Kjeldahl flasks.

## CHAPTER XII.

### BLOOD AND LYMPH.

Blood is composed of four types of form-elements (erythrocytes or red blood corpuscles, leucocytes or white blood corpuscles, blood plates or plaques and blood dust or hæmoconien) held in suspension in a fluid called *blood plasma*. These form-elements compose about 60 per cent of the blood, by weight. Ordinarily blood is a dark red opaque fluid due to the presence of the red blood corpuscles, but through the action of certain substances, such as water, ether, or chloroform, it may be rendered transparent. Blood so altered was formerly said to be *laked*. The term *hæmolysis* is now used in this connection and substances which cause such action are spoken of as *hæmolytic agents*. The hæmolytic process is simply a liberation of the hæmoglobin from the stroma of the red blood corpuscle. Normal blood is alkaline in reaction<sup>1</sup> to litmus, the alkalinity being due principally to sodium carbonate and phosphate. The specific gravity of the blood of adults ordinarily varies between 1.045 and 1.075. It varies somewhat with the sex, the blood of males having a rather higher specific gravity than that of females of the same species. Under pathological conditions also the density of the blood may be very greatly altered. The freezing-point (*d*) of normal blood is about  $-0.56^{\circ}$  C. Variations between  $-0.51^{\circ}$  and  $0.62^{\circ}$  C. may be due entirely to dietary conditions, but if any marked variation is noted it can in most cases be traced to a disordered kidney function. The total amount of blood in the body has been variously estimated at from one-twelfth to one-fourteenth of the body weight. Perhaps  $1/13.5$  is the most satisfactory figure. Abderhalden and Schmidt<sup>2</sup> have recently suggested a unique method for the determination of this value. It is based upon the change in the optical activity of the blood upon injection of a body of known optical activity, such, for example, as dextrin.

Among the most important constituents of blood plasma are the four protein bodies, *fibrinogen*, *nucleoprotein*, *serum globulin* (euglobulin and pseudo-globulin) and *serum albumin*. Plasma contains about 8.2 per cent of solids of which the protein constituents named above constitute approximately 84 per cent and the inorganic constituents (mainly chlorides, phosphates and carbonates) approximately 10 per cent. Among the inorganic constituents sodium chloride predominates. To prevent coagu-

<sup>1</sup> Recently it has been shown by physico-chemical methods that the blood is in reality neutral in reaction.

<sup>2</sup> Abderhalden and Schmidt: *Zeit. physiol. chem.*, 66, 120, 1910.



lation, blood plasma is ordinarily studied in the form of an oxalated or salted plasma. The former may be obtained by allowing the blood to flow from an opened artery into an equal volume of 0.2 per cent ammonium oxalate solution, whereas in the preparation of a salted plasma 10 per cent sodium chloride solution may be used as the diluting fluid.

Fibrinogen is perhaps the most important of the protein constituents of the plasma. It is also found in lymph and chyle as well as in certain exudates and transudates. Fibrinogen possesses the general properties of the globulins, but differs from serum globulin in being precipitated upon half-saturation with sodium chloride. In the process of coagulation of the blood the fibrinogen is transformed into fibrin. This fibrin is one of the principal constituents of the ordinary blood clot.

The nucleoprotein of blood possesses many of the characteristics of serum globulin. In common with this body it is easily soluble in sodium chloride, and is completely precipitated from its solutions upon saturation with magnesium sulphate. It is much less soluble in dilute acetic acid than serum globulin, and its solutions coagulate at  $65^{\circ}$ – $69^{\circ}$  C.

The body formerly called serum globulin is probably not an individual substance. Recent investigations seem to indicate that it may be resolved into two individual bodies called *euglobulin* and *pseudoglobulin*. The euglobulin is practically insoluble in water and may be precipitated in the presence of 28–36 per cent of saturated ammonium sulphate solution. The pseudoglobulin, on the contrary, is soluble in water and is only precipitated by ammonium sulphate in the presence of from 36 to 44 per cent of saturated ammonium sulphate solution.

In common with serum globulin the body known as serum albumin seems also to consist of more than a single individual substance. The so-called serum albumin may be separated into at least two distinct bodies, one capable of crystallization, the other an amorphous body. The solution of either of these bodies in water gives the ordinary albumin reactions. The coagulation temperature of the serum albumin mixture as it occurs in serum or plasma varies from  $70^{\circ}$  to  $85^{\circ}$  C. according to the reaction of the solution and its content of inorganic material. Serum albumin differs from egg albumin in being more lævorotatory, in being rendered less insoluble by alcohol, and in the fact that when precipitated by hydrochloric acid it is more easily soluble in an excess of the reagent.

When blood coagulates and the usual clot forms, a light yellow fluid exudes. This is blood *serum*. It differs from blood plasma in containing a large amount of *fibrin ferment*, a body of great importance in the coagulation of the blood, and also in possessing a lower protein content. The protein material present in plasma and not found in serum is the fibrinogen which is transformed into fibrin in the process of coagulation and

removed. The specific gravity of the serum of human blood varies between 1.026 and 1.032. If blood be drawn into a vessel and allowed to remain without stirring or agitation of any sort the major portion of the red corpuscles will sink away from the upper surface, causing this portion of the clot to assume a lighter color due to the predominance of leucocytes. This light colored portion of the clot is called the "buffy coat."

Beside the protein constituents already mentioned, other bodies which are found in both the plasma and serum are the following: *Sugar* (dextrose), *fat*, *enzymes*, *lecithin*, *cholesterol* and its esters, *gases*, *coloring-matter* (lutein or lipochrome) and *mineral substances*. In addition to these bodies the following substances have been detected in normal human blood: *Creatine*, *carbamic acid*, *hippuric acid*, *paralactic acid*, *urea* and *uric acid* (urates). Some of the *pathological constituents* of blood are *proteoses*, *leucine*, *tyrosine* and other amino acids, *biliary constituents* and *purine bodies*.

Bywaters<sup>1</sup> reports the presence of a glycoprotein in blood serum. This he has termed *seromuroid*.

There has been considerable controversy regarding the form of the erythrocytes or red blood corpuscles of human blood. It is claimed by some investigators that the cells are *bell-shaped* or *cup-shaped*. As the erythrocytes occur normally *in the circulation*, however, they are probably thin, non-nucleated, biconcave discs. When examined singly under the microscope, they possess a pale greenish-yellow color (see Plate IV, opposite), whereas when grouped in large masses a reddish tint is noted.

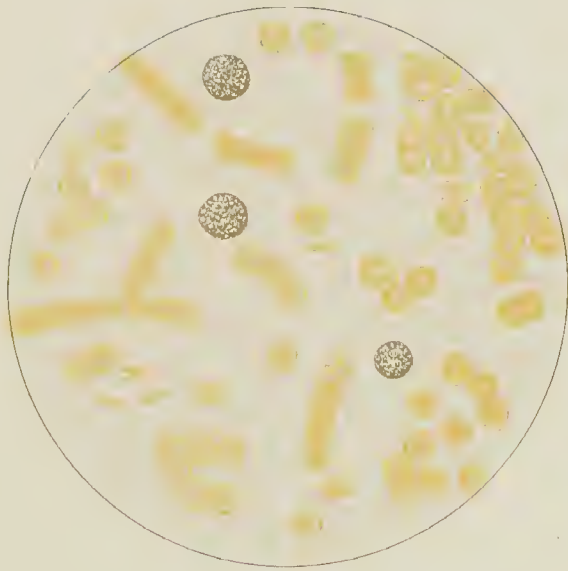
The blood of most mammals contains erythrocytes similar in form to those of human blood. In the blood of birds, fishes, amphibians and reptiles the erythrocytes are ordinarily more or less elliptical, biconvex and possess a nucleus. The erythrocytes vary in size with the different animals. The average diameter of the erythrocytes of blood from various species is given in the following table:<sup>2</sup>

Elephant.....	$\frac{1}{2758}$	of an inch.
Guinea-pig.....	$\frac{1}{3223}$	of an inch.
Man.....	$\frac{1}{8250}$	of an inch.
Monkey.....	$\frac{1}{3382}$	of an inch.
Dog.....	$\frac{1}{3581}$	of an inch.
Rat.....	$\frac{1}{8052}$	of an inch.
Rabbit.....	$\frac{1}{3853}$	of an inch.
Mouse.....	$\frac{1}{3745}$	of an inch.
Lion.....	$\frac{1}{4145}$	of an inch.
Ox.....	$\frac{1}{4279}$	of an inch.
Horse.....	$\frac{1}{4243}$	of an inch.
Pig.....	$\frac{1}{4333}$	of an inch.
Cat.....	$\frac{1}{4372}$	of an inch.
Sheep.....	$\frac{1}{4012}$	of an inch.
Goat.....	$\frac{1}{8134}$	of an inch.
Musk-deer.....	$\frac{1}{12825}$	of an inch.

<sup>1</sup> Bywaters: *Biochemische Zeitschrift*, 15, 322, 1909.

<sup>2</sup> Wormley's *Micro-Chemistry of Poisons*, second edition, p. 733.

PLATE IV.



NORMAL ERYTHROCYTES AND LEUCOCYTES.



The erythrocytes from whatever source obtained, consist essentially of two parts, the *stroma* or protoplasmic tissue and its enclosed pigment, *hæmoglobin*. For human blood the number of erythrocytes present in the fluid as obtained from well-developed males in good physical condition is about 5,500,000 per cubic millimeter.<sup>1</sup> The normal content of the blood of adult females is from 4,000,000 to 4,500,000 per cubic millimeter. The number of erythrocytes varies greatly under different conditions. For instance the number may be increased after the transfusion of blood of the same species of animal; by residing in a high altitude; or as a result of strenuous physical exercise continued over a short period of time. An increase is also noted in starvation; after partaking of food; after cold or hot baths; after massage, as well as after the administration of certain drugs and accompanying certain diseases, such as cholera, diarrhœa, dysentery and yellow atrophy of the liver. A decrease in the number occurs in the different forms of anæmia. The number has been known to increase to 7,040,000 per cubic millimeter as a result of physical exercise, while 11,000,000 per cubic millimeter have been noted in cases of polycythæmia and increases nearly as great in cyanosis. The number has been known to decrease to 500,000 per cubic millimeter or lower in pernicious anæmia.

Erythrocytes possess the property, when properly treated, of "clumping" together in masses and precipitating, producing so-called *agglutination*. Cells other than erythrocytes (*e. g.*, bacteria) possess this property. When spoken of in connection with the blood such action is termed *hæmagglutination*. A substance which will bring about hæmagglutination is said to contain *hæmagglutinins*. These hæmagglutinins are particularly abundant in the vegetable kingdom.<sup>2</sup> For a demonstration of hæmagglutination see page 208.

Oxyhæmoglobin, the coloring matter of the blood, is a conjugated protein. Through treatment with hydrochloric acid it may be split into a protein body called *globin*, and *hæmochromogen*, an iron-containing pigment. The latter body is rapidly transformed into *hæmatin* in the presence of oxygen, and this in turn gives place to hæmatin-hydrochloride or *hæmin* (Figs. 59 and 60, page 211). The pigment of arterial blood is for the most part loosely combined with oxygen and is termed *oxyhæmoglobin*, whereas the pigment of venous blood is principally hæmoglobin (so-called *reduced hæmoglobin*). Oxyhæmoglobin is the oxygen-carrier of the body and belongs to the class of bodies known as respiratory pig-

<sup>1</sup> This statement is based upon observations made upon the blood of athletes in training. See Hawk: *Amer. Jour. Physiol* 1904. It is generally stated in text-books that the blood of males contains about 5,000,000 per cubic millimeter.

<sup>2</sup> Mendel: *Archivio di fisiologia*, 7, 168, 1909; also Schneider: *Journal of Biological Chem.*, 11, 47, 1912.



FIG. 51.—OXYMYOGLOBIN CRYSTALS FROM BLOOD OF THE GUINEA-PIG.  
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

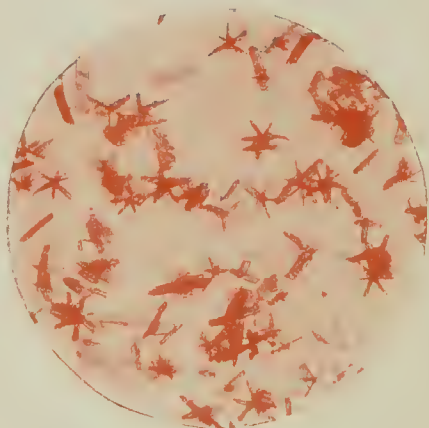


FIG. 52.—OXYMYOGLOBIN CRYSTALS FROM BLOOD OF THE RAT.  
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

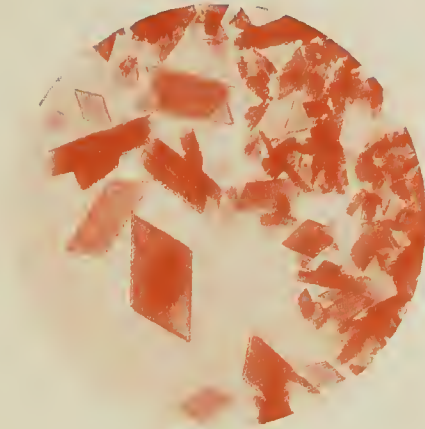


FIG. 53.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE HORSE.  
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

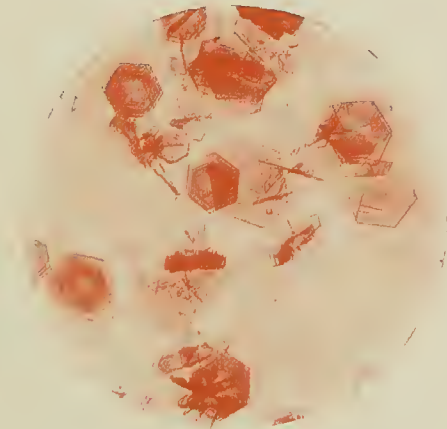


FIG. 54.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE SQUIRREL.  
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

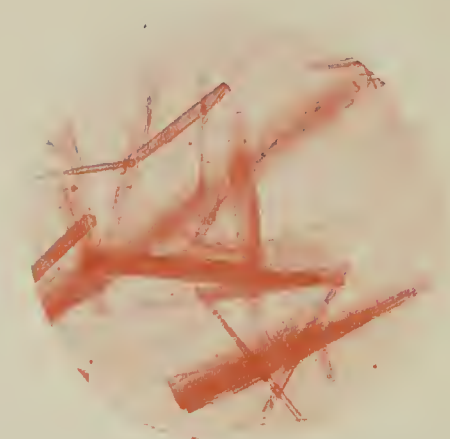


FIG. 55.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE DOG.  
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University  
of Pennsylvania.

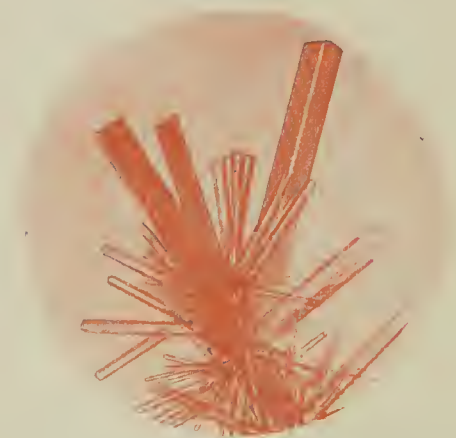


FIG. 56.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE CAT.  
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University  
of Pennsylvania.





FIG. 57.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE NECTURUS. Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.<sup>1</sup>

ments. It is held within the stroma of the erythrocyte. The reduction of oxyhæmoglobin to form hæmoglobin (so-called *reduced hæmoglobin*) occurs in the capillaries. Oxyhæmoglobin may be crystallized and a specific form of crystal obtained from the blood of each individual species (see Figs. 51 to 57, pages 198 to 201). This fact seems to indicate that there are many varieties of oxyhæmoglobin. The interesting findings of Reichert and Brown are of great value in this connection. These investigators prepared oxyhæmoglobin crystals from the blood of over *one hundred* species of animal and subsequently studied the characteristics of the crystals very minutely from the standpoint of crystallography. Their findings may prove of importance from the standpoint of heredity and the origin of species. They emphasize the following facts:

1. Crystals from all species of a certain genus have certain characteristics in general. Crystals from different genera, however, exhibit marked differences in *system*, *axial ratios*, etc.

2. Crystals of different species of a genus may generally be differentiated by difference in the angles.

3. The oxyhæmoglobin of some species crystallizes in several types of crystals in the same preparation. Generally the crystals first formed belong to a system of a lower grade of symmetry than those formed later. When such different types of crystals occur they may be arranged in isomorphous series.

4. Certain definite angles recur in the crystals from the blood of

<sup>1</sup> The micro-photographs of oxyhæmoglobin (see pages 198-201) and hæmin (see page 211) are reproduced through the courtesy of Professors E. T. Reichert and Amos P. Brown, of the University of Pennsylvania, who are investigating the crystalline forms of biochemic substances.

various species of animal, although the zoölogical connection may be remote and the crystals belong to different systems.

5. The constant recurrence of certain types of "twinning" in all the crystalline forms was observed.

6. Differences have been observed in the crystalline form of oxy-hæmoglobin and hæmoglobin from the blood of the same species in certain cases.

The following bodies may be derived from hæmoglobin, and each possesses a specific spectrum which serves as an aid in its detection and identification: Oxyhæmoglobin, methæmoglobin, carbon-monoxide hæmoglobin, nitric-oxide hæmoglobin, hæmochromogen, hæmatin, acid-hæmatin, alkali-hæmatin and hæmatoporphyrin (see Absorption Spectra, Plates I and II).

The white corpuscles (or leucocytes) of human blood differ from the red corpuscles (or erythrocytes) in many particulars, such as being somewhat larger in size, in containing at least a single nucleus and in possessing amœboid movement (see Plate IV, opposite page 196). They are typical animal cells and therefore contain the following bodies which are customarily present in such cells: *Proteins, fats, glycogen, purine bodies, enzymes, phosphatides, lecithin, cholesterol, inorganic salts and water.* Compound proteins make up the chief part of the protein quota of leucocytes, the nucleo-proteins predominating. Of the enzymes present the proteolytic are the most important. It is claimed<sup>1</sup> that there are two proteolytic enzymes in leucocytes, one active in alkaline solution and present in the polynuclear cells<sup>2</sup> and the other active in acid medium and present in mononuclear cells. It is claimed that the granular leucocytes originate in the bone marrow, whereas the non-granular leucocytes (lymphocytes) have a lymphatic origin (lymph glands or lymphoid tissue); this matter of origin is uncertain. The normal number of leucocytes in human blood varies between 5000 and 10,000 per cubic millimeter. The ratio between the leucocytes and erythrocytes is about 1:350-500. A *leucocytosis* is said to exist when the number of leucocytes is increased for any reason. Leucocytoses may be divided into two general classes, the *physiological* and the *pathological*. Under the physiological form would be classed those leucocytoses accompanying pregnancy, parturition and digestion, as well as those due to mechanical and thermal influences. The leucocytoses spoken of as pathological are the inflammatory, infectious, post-hæmorrhagic, toxic and experimental forms as well as the type of leucocytosis which accompanies malignant disease.

<sup>1</sup> Opie: *Jour. of Experimental Med.*, 8; Opie and Barker: *Ibid.*, 9.

<sup>2</sup> For discussion of different types of leucocytes, see "Da Costa's Clinical Hematology" or some similar volume.

The blood plates (platelets or plaques) are round or oval, colorless discs which possess a diameter about one-third as great as that of the erythrocytes. Upon treatment with certain reagents, *e. g.*, artificial gastric juice, they may be separated into a homogeneous, non-refractive portion and a granular, refractive portion. The blood plates are probably associated in some way with the coagulation of the blood. This relationship is not well understood at present.

The hæmoconein or so-called "blood dust" is made up of round granules which usually have a diameter somewhat less than one micron. The serum of normal as well as of pathological blood contains these granules. They were first described by Müller to whom they appeared as highly refractile granules possessed of Brownian movement. The "blood dust" is apparently not concerned with the coagulation of the blood. The granules are insoluble in alcohol, ether and acetic acid and are not blackened by osmic acid. According to Müller, the granules making up the so-called "blood dust" constitute a new organized constituent of the blood, whereas other investigators believe them to be merely free granules from certain of the forms of leucocytes. In common with blood plates the "blood dust" possesses no clinical significance.

The processes involved in the coagulation of the blood are not fully understood. Several theories have been advanced and each has its adherents. The theory which appears to be fully as firmly founded upon experimental evidence as any is the following: Blood contains a zymogen called *prothrombin* which combines with the calcium salts present to form an enzyme known as *thrombin* or *fibrin-ferment*. When freshly drawn blood comes in contact with the air the fibrin-ferment at once acts upon the fibrinogen present and gives rise to the formation of *fibrin*. This fibrin forms in shreds throughout the blood mass and, holding the form elements of the blood within its meshes, serves to produce the typical *blood clot*. The fibrin shreds gradually contract, the whole clot assumes a jelly-like appearance and the yellowish serum exudes. If, immediately upon the withdrawal of blood from the body, the fluid be rapidly stirred or thoroughly "whipped" with a bundle of coarse strings, twigs or a specially constructed beater, the fibrin shreds will not form in a network throughout the blood mass but instead will cling to the device used in beating. In this way the fibrin may be removed and the remaining fluid is termed *defibrinated* blood. The above theory of the coagulation of the blood may be stated briefly as follows:

I. Prothrombin + Calcium Salts = Thrombin (or Fibrin-ferment).

II. Thrombin (or Fibrin-ferment) + Fibrinogen = Fibrin.

Howell<sup>1</sup> has very recently suggested an ingenious modification of the

<sup>1</sup> Howell: *American Journal of Physiology*, 29, 187, 1911.

above theory. He says "In the circulating blood we find as constant constituents, *fibrinogen*, *prothrombin*, *calcium salts* and *antithrombin*. The last-named substance holds the prothrombin in combination and thus prevents its conversion or activation to thrombin. When the blood is shed, the disintegration of the corpuscles (platelets) furnishes material (thromboplastin) which combines with the antithrombin and liberates the prothrombin; the latter is then activated by the calcium and acts on the fibrinogen. According to this view the actual process of coagulation involves only three factors, fibrinogen, prothrombin and calcium. These three factors exist normally in the circulating blood, but are prevented from reacting by the presence of antithrombin."

Among the medico-legal tests for blood are the following: (1) Microscopical identification of the erythrocytes, (2) spectroscopic identification of blood solutions, (3) the guaiac test, (4) the benzidine reaction, (5) preparation of hæmin crystals. Of these five tests the two last named are generally considered to be the most satisfactory. They give equally reliable results with fresh blood and with blood from clots or stains of long standing, provided the latter have not been exposed to a high temperature, or to the rays of the sun for a long period. The technic of the tests is simple and the formation of the dark brown or chocolate colored crystals of hæmin or the production of the green or blue color with benzidine is indisputable proof of the presence of blood in the fluid, clot or stain examined. The weak point of the tests, medico-legally, lies in the fact that they do not differentiate between human blood and that of certain other species of animal.

The guaiac test (see page 209), although generally considered less accurate than the hæmin test, is really a more delicate test than the hæmin test if properly performed. One of the most common mistakes in the manipulation of this test is the use of a guaiac solution which is too concentrated and which, when brought into contact with the aqueous blood solution, causes the separation of a voluminous precipitate of a resinous material which may obscure the blue coloration: this is particularly true of the test when used for the examination of blood stains. A solution of guaiac made by dissolving 1 gram of the resin in 60 c.c. of 95 per cent alcohol is very satisfactory for general use. The test is frequently objected to upon the ground that various other substances, *e. g.*, milk, pus, saliva, etc., respond to the test and that it cannot therefore be considered a specific test for blood and is of value only in a negative sense. We have demonstrated to our own satisfaction, however, that milk many times gives the blue color upon the addition of an alcoholic solution of guaiac resin without the addition of hydrogen peroxide or old turpentine. Buckmaster has advocated the use of an alcoholic solution of guaiac-

conic acid instead of an alcoholic solution of guaiac resin. He claims that he was able to produce the blue color upon the addition of the guaiaconic acid to milk *only* when the sample of milk tested was brought from the country in *sterile bottles*, and further, that no sample of London milk which he examined responded to the test. In the application of the guaiac test to the detection of blood, he states that he was able to detect *laked blood* when present in the ratio 1:5,000,000 and *unlaked blood* when present in the ratio 1:1,000,000. This author considers the guaiac test to be far more trustworthy than is generally believed.

Up to within recent times it has been impossible to make an absolute differentiation of human blood. Recently, however, the so-called "biological" blood test has made such a differentiation possible. This test, known as the Bordet reaction, is founded upon the fact that the blood serum of an animal into which has been injected the blood of another animal of different species develops the property of agglutinating and dissolving erythrocytes *similar to those injected*, but exerts this influence upon the blood from *no other species*. The antiserum used in this test is prepared by injecting rabbits with 5-10 c.c. of human defibrinated blood, at intervals of about four days until a total of between 50 and 80 c.c. has been injected. After a lapse of one or two weeks the animal is bled, the serum collected, placed in sterile tubes and preserved for use as needed. In examining any specific solution for human blood it is simply necessary to combine the antiserum and the solution under examination in the proportion of 1:100 and place the mixture at 37° C. If human blood is present in the solution a turbidity will be noted and this will change within three hours to a distinctly flocculent precipitate. This antiserum will react thus with no other known substance.

Lymph may be considered as the "middle man" in the transactions between blood and tissues. It is the medium by which the nutritive material and oxygen transported by the blood for the tissues is brought into intimate contact with those tissues and thus utilized. In the further fulfillment of its function, the lymph bears from the tissues water, salts and the products of the activity and catabolism of the tissues and passes these into the blood. Lymph, therefore, exercises the function of a "go-between" for blood and tissues. It bathes every active tissue of the animal body, and is believed to have its origin partly in the blood and partly in the tissues.

In chemical characteristics, lymph resembles blood plasma. In fact, it has been termed "blood without its red corpuscles." Lymph from the thoracic duct of a fasting animal or from a large lymphatic vessel of a well-nourished animal is of a variable color (colorless, yellowish or slightly reddish) and alkaline in reaction to litmus. It contains fibrinogen, fibrin

ferment and leucocytes and coagulates slowly, the clot being less firm and bulky than the blood clot. Serum albumin and serum globulin are both present in lymph, the albumin predominating in a ratio of about 3 or 4 : 1. The principle inorganic salts are sodium salts (chloride and carbonate), whereas the phosphates of potassium, calcium, magnesium and iron are present in smaller amount.

Substances which stimulate the flow of lymph are termed *lymphagogues*. Such substances, as sugar, urea, certain salts (especially sodium chloride) peptone, egg albumin, extracts of dogs' liver and intestine, crab muscles and blood leeches are included in this class.

In a fasting animal, the lymph coming from the intestine is a clear, transparent fluid possessing the characteristics already outlined. After a meal containing fat has been ingested, this intestinal lymph is white or "milky." This is termed *chyle* and is essentially lymph possessing an abnormally high (5-15 per cent) content of emulsified fat. This chyle is absorbed by the lacteals of the intestine and transported to the lower portion of the thoracic duct. Apart from the fat value, the composition of lymph and chyle are similar.

## EXPERIMENTS ON BLOOD.

### I. Defibrinated Ox-blood.

1. **Reaction.**—Moisten red and blue litmus papers with 10 per cent sodium chloride solution and test the reaction of the defibrinated blood. Test by congo-red paper also.

2. **Microscopical Examination.**—Examine a drop of defibrinated blood under the microscope. Compare the objects you observe with Plate IV, opposite page 196. Repeat the test with a drop of your own blood.

3. **Specific Gravity.**—Determine the specific gravity of defibrinated blood by means of an ordinary specific gravity spindle. Compare this result with the specific gravity as determined by Hammerschlag's method in the next experiment.

4. **Specific Gravity by Hammerschlag's Method.**—Fill an ordinary urinometer cylinder about one-half full of a mixture of chloroform and benzene, having a specific gravity of approximately 1.050. Into this mixture allow a drop of the blood under examination to fall from a pipette or directly from the finger in case fresh blood is being examined. Care must be taken not to use too large a drop of blood and to keep the drop from coming in contact with the walls of the cylinder. If the blood drop sinks to the bottom of the vessel, thus showing it to be of higher specific gravity than the surrounding fluid, add chloroform until the blood drop remains

suspended in the mixture. Stir carefully with a glass rod after adding the chloroform. If the blood drop rises to the surface upon being introduced into the mixture, thus showing it to be of lower specific gravity than the surrounding fluid, add benzene until the blood drop remains suspended in the mixture. Stir with a glass rod after the benzene is added. After the blood drop has been brought to a suspended position in the mixture by means of one or more additions of chloroform and benzene this final mixture should be filtered through muslin and its specific gravity accurately determined. What is the specific gravity of the blood under examination?

5. **Tests for Various Constituents.**—Place 10 c.c. of defibrinated blood in an evaporating dish, dilute with 100 c.c. of water and heat to boiling. Is there any coagulation, and if so what bodies form the coagulum? At the boiling-point acidulate slightly with dilute acetic acid. Filter. The filtrate should be clear and the coagulum dark brown. Reserve this coagulum. What body gives the coagulum this color? Evaporate the filtrate to about 25 c.c., filtering off any precipitate which may form in the process. Make the following tests upon the filtrate:

(a) *Fehling's Test.*—Test for sugar according to directions given on page 32.

(b) *Chlorides.*—To a small amount of the filtrate in a test-tube add a few drops of nitric acid and a little silver nitrate. In the presence of chloride, a white precipitate of silver chloride will form.

(c) *Phosphates.*—Test for phosphates by nitric acid and molybdic solution according to directions given on page 64.

(d) *Proteose and Peptone.*—Test a small amount of the solution for proteose and peptone by saturating with ammonium sulphate according to directions given on page 120.

(e) *Crystallization of Sodium Chloride.*—Place the remainder of the filtrate in a watch glass and evaporate it on a water-bath. Examine the crystals under the microscope and compare them with those in Fig. 61, page 213.

6. **Test for Iron.**—Incinerate a small portion of the coagulum from the last experiment (5) in a porcelain crucible. Cool, dissolve the residue in dilute hydrochloric acid and test for iron by potassium ferrocyanide or ammonium thiocyanate. Which of the constituents of the blood contains the iron?

7. **Hæmolysis ("Laky Blood").**—Note the opacity of ordinary defibrinated blood. Place a few cubic centimeters of this blood in a test-tube and add water, a little at a time, until the blood is rendered transparent. *Hæmolysis* has taken place. How does the water act in causing this transparency? Examine a drop of hæmolyzed blood under the microscope. How does its microscopical appearance differ from that of

unaltered blood? What other agents may be used to bring about hæmolytic?

8. **Osmotic Pressure.**—Place a few cubic centimeters of blood in each of three test-tubes. Hæmolyze the blood in the first tube according to directions given in the last experiment (7): add an equal volume of *isotonic* (0.9 per cent) sodium chloride to the blood in the second tube, and an equal volume of 10 per cent sodium chloride to the blood in the third tube. Mix thoroughly by shaking and after a few moments examine a drop from each of the three tubes under the microscope (see Figs. 58 and 120, below and p. 377). What do you find and what is your explanation from the standpoint of osmotic pressure?

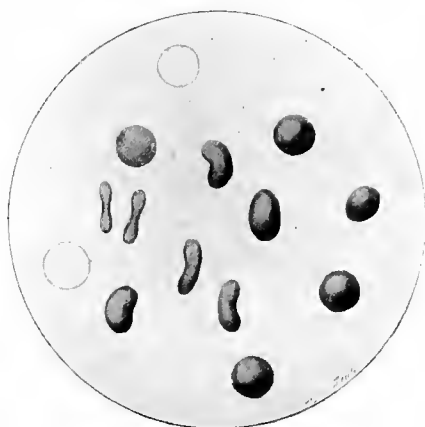


FIG. 58.—EFFECT OF WATER ON ERYTHROCYTES.

9. **Hæmagglutination.**—The common garden bean, such as the Scarlet Runner,<sup>1</sup> contains a protein substance which exhibits the interesting property of causing a clumping or agglutination of red blood corpuscles.<sup>2</sup>

Dilute defibrinated blood<sup>3</sup> ten times with physiological sodium chloride solution (0.9 per cent) and place 1 c.c. in each of three small test-tubes.

Grind three beans in a coffee mill, or with mortar and pestle to a fine meal and extract for a few minutes with 0.9 per cent sodium chloride solution. Filter and add 0.05 c.c. (about 2–3 drops) of the filtered

<sup>1</sup> The Scarlet Runner is a familiar variety purchasable in every seed store. Ricin a protein constituent of the castor bean also possesses pronounced agglutinating properties. Because of its poisonous nature it is, however, not suitable for use in class experiments.

<sup>2</sup> Mendel: *Archivio di fisiologia*, 7, 168, 1909; Schneider: *Journal Biol. Chem.*, 11, 47, 1912.

<sup>3</sup> Rabbit's blood is especially desirable (Mendel: *Loc. cit.*) and may be obtained for the purpose by bleeding from a small cut on the animal's ear and defibrinating.



extract to the first of the blood tubes; 0.01 c.c. to the second; and 0.05 of 0.9 per cent sodium chloride solution to the third.

Invert each tube to mix the contents thoroughly, and note the rapid agglutination, and precipitation of the blood corpuscles in the first tube, a less rapid agglutination in the second, while the third or control tube remains unaltered. In one-half hour the corpuscles in the first tube often are *packed solid* and one is able to pour off *perfectly clear serum*.

If the remainder of the bean extract is boiled for a few minutes, the coagulum filtered out and 0.05 c.c. of the filtrate added to the control tube, still no agglutination occurs, indicating that the hæmagglutinin has been destroyed or removed by the boiling.

10. **Diffusion of Hæmoglobin.**—Prepare some *hæmolyzed* ("laky") blood, thus liberating the hæmoglobin from the erythrocytes. Test the diffusion of the hæmoglobin by preparing a dialyzer like one of the models shown in Fig. 2, page 30. How does hæmoglobin differ from other well-known crystallizable bodies?

11. **Guaiac Test.**—To 5 c.c. of water in a test-tube add two drops of blood. By means of a pipette drop an alcoholic solution of guaiaic (strength about 1:60)<sup>1</sup> into the resulting mixture until a turbidity is observed and add old turpentine or hydrogen peroxide, drop by drop, until a blue color is obtained. Do any other substances respond in a similar manner to this test? Is a positive guaiaic test a sure indication of the presence of blood?

12. **Schumm's Modification of the Guaiaic Test.**—To about 5 c.c. of the solution under examination<sup>2</sup> in a test-tube add about ten drops of freshly prepared alcoholic solution of guaiaic. Agitate the tube gently, add about 20 drops of old turpentine, subject the tube to a thorough shaking and permit it to stand for about 2–3 minutes. A blue color indicates the presence of blood in the solution under examination. In case there is insufficient blood to yield a blue color under these conditions, a few c.c. of alcohol should be added and the tube gently shaken, where-upon a blue coloration will appear in the upper alcohol-turpentine layer.

A control test should always be made, using water in place of the solution under examination. In the detection of very minute traces of blood only 3–5 drops of the guaiaic solution should be employed.

13. **Adler's Benzidine Reaction.**—This is one of the most delicate of the reactions for the detection of blood. Different benzidine prepara-

<sup>1</sup> Buckmaster advises the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiaic resin.

<sup>2</sup> Alkaline solutions should be made slightly acid with acetic acid, as the blue end-reaction is very sensitive to alkali.

tions vary greatly in their sensitiveness, however. Inasmuch as benzidine solutions change readily upon contact with light it is essential that they be kept in a dark place. The test is performed as follows: To a saturated solution of benzidine in alcohol or glacial acetic acid add an equal volume of 3 per cent hydrogen peroxide and one c.c. of the solution under examination. If the mixture is not already acid render it so with acetic acid, and note the appearance of a green or blue color. A control test should be made substituting water for the solution under examination. The sensitiveness of the benzidine reaction is greater when applied to aqueous solutions than when applied to the urine. According to Ascarelli<sup>1</sup> the benzidine reaction serves to detect blood when present in a dilution of 1:300,000. Walter<sup>2</sup> has also recently shown the test to be very delicate and claims it to be more satisfactory than the guaiac test.

14. **Hæmin Test.**—(a) *Teichmann's Method.*—Place a *very small* drop of blood on a microscopic slide, add a minute grain of sodium chloride<sup>3</sup> and *carefully* evaporate to *dryness* over a *low flame*. Put a cover glass in place, run underneath it a drop of *glacial* acetic acid and *warm gently* until the formation of gas bubbles is noted. Add another drop of glacial acetic acid, cool the preparation, examine under the microscope and compare the crystals with those shown in Figs. 59 and 60, page 211. The hæmin crystals result from the decomposition of the hæmoglobin of the blood. What are the steps involved in this process? The hæmin crystals are also called Teichmann's crystals. Is this an *absolute* test for blood? Is it possible to differentiate between human blood and the blood of other species by means of the hæmin test?

(b) *Atkinson and Kendall's Method.*—Introduce a small amount of the solution under examination into a tube closed at one end, add sodium chloride and glacial acetic acid as in Teichmann's method,<sup>4</sup> fuse or tightly plug the open end of the tube and heat for fifteen minutes in a boiling water-bath.<sup>5</sup> Remove the tube and permit it to cool to room temperature spontaneously. When the tube has cooled, break it open, transfer the contents to a watch glass or small evaporating dish and concentrate on a water-bath until the volume of the fluid in the watch glass or dish has been reduced to a few drops. Transfer a drop of this fluid to a slide, cover with a cover slip, allow the slide to stand for a few minutes and examine it under a microscope. Compare the crystals with those shown in Figs. 59 and 60, page 211. In case crystals of sodium chloride (see Fig. 61, page 213) obstruct the view of the hæmin crystals,

<sup>1</sup> Ascarelli: *Il policlin sez. prat.*, 1909.

<sup>2</sup> Walter: *Deut. med. Woch.*, 36 p. 309.

<sup>3</sup> Buckmaster considers the use of potassium chloride preferable.

<sup>4</sup> Care should be taken not to add too great an excess of these reagents.

<sup>5</sup> This process insures constancy of temperature and strength of reagents.

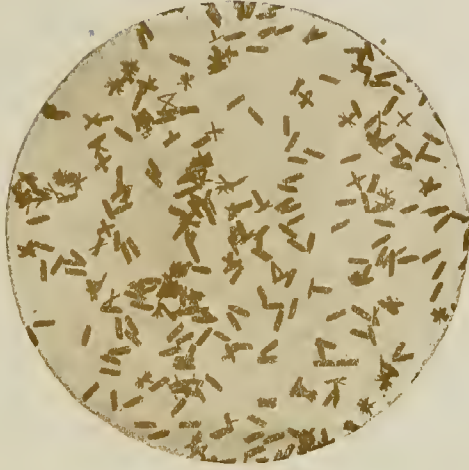


FIG. 59.—HÆMIN CRYSTALS FROM HUMAN BLOOD.

Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.



FIG. 60.—HÆMIN CRYSTALS FROM SHEEP BLOOD.

Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

dissolve the sodium chloride crystals by running a drop of water under the cover slip.

(c) *v. Zeynek and Nencki's Method.*—To 10 c.c. of defibrinated blood add acetone until no more precipitate forms. Filter off the precipitated protein and extract it with 10 c.c. of acetone made acid with 2–3 drops of hydrochloric acid. Place a drop of the resulting colored extract on a slide, immediately place a cover glass in position and examine under the microscope. Upon the evaporation of the acetone, crystals of hæmin will form. Larger crystals may be obtained by evaporating the acetone extract about one-half, transferring it to a stoppered vessel and allowing it to remain overnight.

(d) *Schalfjew's Method.*—Place 20 c.c. of glacial acetic acid in a small beaker and heat to 80° C. Add 5 c.c. of strained defibrinated blood, again bring the temperature to 80° C., remove the flame and allow the mixture to cool. Examine the crystals under the microscope and compare them with those reproduced in Figs. 59 and 60, page 211.

15. **Catalytic Action.**—To about 10 drops of blood in a test-tube add twice the volume of hydrogen peroxide, without shaking. The mixture foams. What is the cause of this phenomenon?

16. **Preparation of Hæmatin.**—Place 100 c.c. of *hæmolyzed (laked)* blood in a beaker and add 95 per cent alcohol until precipitation ceases. What bodies are precipitated? Transfer the precipitate to a flask and boil with 95 per cent alcohol previously acidulated with sulphuric acid. Through the action of the acid the hæmoglobin is split into hæmatin and a protein body called globin. Later the “sulphuric acid ester of hæmatin” is formed, which is soluble in the alcohol. Continue heating until the precipitate is no longer colored, then filter. Partly saturate the filtrate with sodium chloride and warm. In this process the “hydrochloric acid ester of hæmatin” is formed. Filter and dissolve on the filter paper by sodium carbonate. Save this alkaline solution of hæmatin and make a spectroscopic examination later after becoming familiar with the use of the spectroscope. How does the spectrum of oxyhæmoglobin differ from that of the derived *alkali hæmatin*?

17. **Variation in Size of Erythrocytes.**—Prepare two small funnels with filter papers such as are used in quantitative analysis. Moisten each paper with physiological (isotonic) salt solution. Into one funnel introduce a small amount of defibrinated ox blood and into the other funnel allow blood to drop directly from a decapitated frog. Note that the filtrate from the ox blood is colored whereas that from the frog blood is colorless. What deduction do you make regarding the relative size of the erythrocytes in ox and frog blood? Does either filtrate clot? Why?

II. Blood Serum.<sup>1</sup>

1. **Coagulation Temperature.**—Place 5 c.c. of undiluted serum in a test-tube and determine its temperature of coagulation according to the method described on page 106. Note the temperature at which a cloudiness occurs as well as the temperature at which coagulation is complete.

2. **Precipitation by Alcohol.**—To 5 c.c. of serum in a test-tube add twice the amount of 95 per cent alcohol and thoroughly mix by shaking. What is this precipitate? Make a confirmatory test. Test the alcoholic filtrate for protein. Explain the result.

3. **Proteins of Blood Serum.**—Place about 10 c.c. of serum in a small evaporating dish, dilute with 5 c.c. of water and heat to boiling. At the boiling-point acidify slightly with dilute acetic acid. Of what does this coagulum consist? Filter off the coagulum (reserve the filtrate) and test it as follows:

(a) *Millon's Reaction.*—Make the test according to directions given on page 97.

(b) *Hopkins-Cole Reaction.*—Make the test according to directions given on page 98.

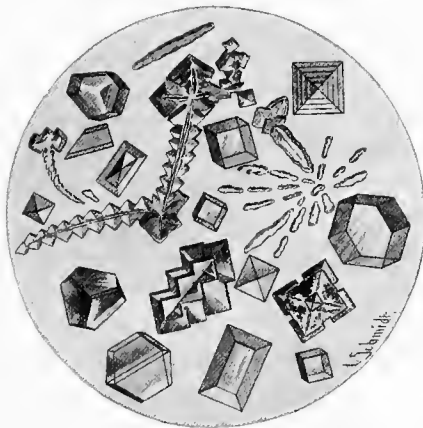


FIG. 61.—SODIUM CHLORIDE.

4. **Sugar in Serum.**—Test a little of the filtrate from Experiment 3 by Fehling's test. What do you conclude?

5. **Detection of Sodium Chloride.**—(a) Test a little of the filtrate from Experiment 3 for chlorides, by the use of nitric acid and silver nitrate. (b) Evaporate 5 c.c. of the filtrate from Experiment 3 in a watch glass on a water-bath. Examine the crystals and compare them with those reproduced in Fig. 61, above.

<sup>1</sup> For directions as to preparation of serum, see Appendix.

6. **Separation of Serum Globulin and Serum Albumin.**—Place 10 c.c. of blood serum in a small beaker and saturate with magnesium sulphate. What is this precipitate? Filter it off and acidify the filtrate slightly with acetic acid. What is this second precipitate? Filter this precipitate off and test the filtrate by the biuret test. What do you conclude?

### III. Blood Plasma.

1. **Preparation of Oxalated Plasma.**—Allow arterial blood to run into an equal volume of 0.2 per cent ammonium oxalate solution.

2. **Preparation of Fibrinogen.**—To 25 c.c. of oxalated plasma add an equal volume of saturated sodium chloride solution. Note the precipitation of fibrinogen. Filter off the precipitate (reserve the filtrate) and test it by a protein color test (see page 97).

3. **Effect of Calcium Salts.**—Place a small amount of oxalated plasma in a test-tube and add a few drops of a 2 per cent calcium chloride solution. What occurs? Explain it.

4. **Preparation of Salted Plasma.**—Allow arterial blood to run into an equal volume of a saturated solution of sodium sulphate or a 10 per cent solution of sodium chloride. Keep the mixture in a cool place for about twenty-four hours.

5. **Effect of Dilution.**—Place a few drops of salted plasma in a test-tube and dilute it with 10–15 volumes of water. What do you observe? Explain it.

6. **Crystallization of Oxyhæmoglobin.** *Reichert's Method.*—Add to 5 c.c. of the blood of the dog, horse, guinea-pig, or rat, before or after laking, or defibrinating, from 1 to 5 per cent of ammonium oxalate *in substance*. Place a drop of this oxalated blood on a slide and examine under the microscope. The crystals of oxyhæmoglobin will be seen to form at once near the margin of the drop, and in a few minutes the entire drop may be a solid mass of crystals. Compare the crystals with those shown in Figs. 51 to 57, pages 198 to 201.

### IV. Fibrin.

1. **Preparation of Fibrin.**—Allow blood to flow directly from the animal into a vessel and rapidly *whip* it by means of a bundle of twigs, a mass of strong cords, or a specially constructed beater. If a pure fibrin is desired it is not best to attempt to manipulate a large volume of blood at one time. After the fibrin has been collected it should be freed from any adhering blood clots and washed in water to remove further traces of blood. The pure product should be very light in color. It may be preserved under glycerol, dilute alcohol, or chloroform water.

2. **Solubility.**—Try the solubility of small shreds of freshly prepared fibrin in the usual solvents.

3. **Millon's Reaction.**—Make the test according to directions given on page 97.

4. **Hopkins-Cole Reaction.**—Make the test according to directions given on page 98.

5. **Biuret Test.**—Make the test according to directions given on page 98.

#### V. Detection of Blood in Stains on Cloth, Etc.

1. **Identification of Corpuscles.**—If the stain under examination is on cloth a portion should be extracted with a few drops of glycerol or physiological (0.9 per cent) sodium chloride solution. A drop of this solution should then be examined under the microscope to determine if corpuscles are present.

2. **Tests on Aqueous Extract.**—A second portion of the stain should be extracted with a small amount of water and the following tests made upon the aqueous extract:

(a) *Hæmochromogen.*—Make a small amount of the extract alkaline by potassium hydroxide or sodium hydroxide, and heat until a brownish-green color results. Cool and add a few drops of ammonium sulphide or Stokes' reagent (see page 216) and make a spectroscopic examination. Compare the spectrum with that of hæmochromogen (see Absorption Spectra, Plate II).

(b) *Hæmin Test.*—Make this test upon a small drop of the aqueous extract according to the directions given on page 210.

(c) *Guaiac Test.*—Make this test on the aqueous extract according to the directions given on page 209. The guaiac solution may also be applied directly to the stain without previous extraction in the following manner: Moisten the stain with water, and after allowing it to stand several minutes, add an alcoholic solution of guaiac (strength about 1:60) and a little hydrogen peroxide or old turpentine. The customary blue color will be observed in the presence of blood.

(d) *Benzidine Reaction.*—Make this test according to directions given on p. 209.

(e) *Acid Hæmatin.*—If the stain fails to dissolve in water extract with acid alcohol and examine the spectrum for absorption bands of acid hæmatin (see Absorption Spectra, Plate II).

#### VI. Spectroscopic Examination of Blood.

(For Absorption Spectra see Plates I. and II.)

Either the *angular*-vision spectroscope (Figs. 63 and 64, page 217) or the *direct*-vision spectroscope (Fig. 62, page 216) may be used in making

the spectroscopic examination of the blood. For a complete description of these instruments the student is referred to any standard text-book of physics.

1. **Oxyhæmoglobin.**—Examine dilute (1:50) defibrinated blood spectroscopically. Note the broad absorption-band between D and E. Continue the dilution until this single broad band gives place to two narrow bands, the one nearer the D line being the narrower. These are the typical absorption-bands of oxyhæmoglobin obtained from dilute solutions of blood. Now dilute the blood *very freely* and note that the bands gradually become more narrow and, if the dilution is sufficiently great, they finally entirely disappear.



FIG. 62.—DIRECT-VISION SPECTROSCOPE.

2. **Hæmoglobin (so-called *Reduced Hæmoglobin*).**—To blood which has been diluted sufficiently to show well-defined oxyhæmoglobin absorption-bands add a small amount of Stokes' reagent.<sup>1</sup> The blood immediately changes in color from a bright red to violet-red. The oxyhæmoglobin has been reduced through the action of Stokes' reagent and hæmoglobin (so-called *reduced hæmoglobin*) has been formed. This has been brought about by the removal of some of the loosely combined oxygen from the oxyhæmoglobin. Examine this hæmoglobin spectroscopically. Note that in place of the two absorption bands of oxyhæmoglobin we now have a single broad band lying almost entirely between D and E. This is the typical spectrum of hæmoglobin. If the solution showing this spectrum be shaken in the air for a few moments it will again assume the bright red color of oxyhæmoglobin and show the characteristic spectrum of that pigment.

3. **Carbon Monoxide Hæmoglobin.**—The preparation of this pigment may be easily accomplished by passing ordinary illuminating gas<sup>2</sup> through defibrinated ox-blood. Blood thus treated assumes a brighter tint (carmine) than that imparted by oxyhæmoglobin. In very dilute solution oxyhæmoglobin appears yellowish-red whereas carbon monoxide hæmoglobin under the same conditions appears bluish-red. Examine the

<sup>1</sup> Stokes' reagent is a solution containing 2 per cent ferrous sulphate and 3 per cent tartaric acid. When needed for use a small amount should be placed in a test-tube and ammonium hydroxide added until the precipitate which forms on the first addition of the hydroxide has entirely dissolved. This produces *ammonium ferrotartrate* which is a reducing agent.

<sup>2</sup> The so-called water gas with which ordinary illuminating gas is diluted contains usually as much as 20 per cent of carbon monoxide (CO).



carbon monoxide hæmoglobin solution spectroscopically. Observe that the spectrum of this body resembles the spectrum of oxyhæmoglobin in showing two absorption-bands between D and E. The bands of carbon

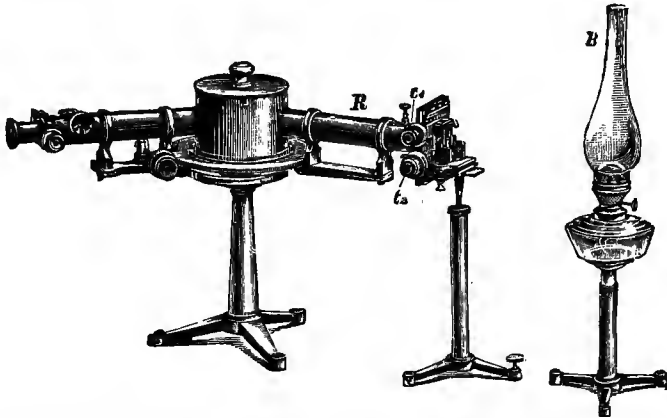


FIG. 63.—ANGULAR-VISION SPECTROSCOPE ARRANGED FOR ABSORPTION ANALYSIS.

monoxide hæmoglobin, however, are somewhat nearer the violet end of the spectrum. Add some Stokes' reagent to the solution and again examine spectroscopically. Note that the position and intensity of the absorption-bands remain unaltered.

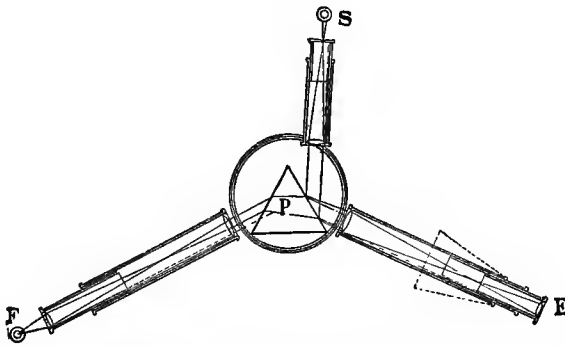


FIG. 64.—DIAGRAM OF ANGULAR-VISION SPECTROSCOPE. (Long.)

The white light *F* enters the collimator tube through a narrow slit and passes to the prism, *P*, which has the power of refracting and dispersing the light. The rays then pass to the double convex lens of the ocular tube and are deflected to the eye-piece *E*. The dotted lines show the magnified virtual image which is formed. The third tube contains a scale whose image is reflected into the ocular and shown with the spectrum. Between the light *F* and the collimator slit is placed a cell to hold the solution undergoing examination.

The following is a delicate *chemical* test for the detection of carbon monoxide hæmoglobin:

*Tannin Test.*—Divide the blood to be tested into two portions and dilute each with four volumes of distilled water. Place the diluted blood

mixtures in two small flasks or large test-tubes and add 20 drops of a 10 per cent solution of potassium ferricyanide.<sup>1</sup> Allow both solutions to stand for a few minutes, then stopper the vessels and shake one vigorously for 10–15 minutes, occasionally removing the stopper to permit air to enter the vessel.<sup>2</sup> Add 5–10 drops of ammonium sulphide (yellow) and 10 c.c. of a 10 per cent solution of tannin to each flask. The contents of the shaken flask will soon exhibit the formation of a dirty olive green precipitate, whereas the flask which was not shaken and which, therefore, still contains carbon monoxide hæmoglobin, will exhibit a bright red precipitate, characteristic of carbon monoxide hæmoglobin. This test is more delicate than the spectroscopic test and serves to detect the presence of as low a content as 5 per cent of carbon monoxide hæmoglobin.

4. **Neutral Methæmoglobin.**—Dilute a little defibrinated blood (1 : 10) and add a few drops of a freshly prepared 10 per cent solution of potassium ferricyanide. Shake this mixture and observe that the bright red color of the blood is displaced by a brownish red. Now dilute a little of this solution and examine it spectroscopically. Note the single, very dark absorption-band lying to the left of D, and, if the dilution is sufficiently great, also observe the two rather faint bands lying between D and E in somewhat similar positions to those occupied by the absorption bands of oxyhæmoglobin. Add a few drops of Stokes' reagent to the methæmoglobin solution while it is in position before the spectroscope and note the immediate appearance of the oxyhæmoglobin spectrum which is quickly followed by that of hæmoglobin.

5. **Alkaline Methæmoglobin.**—Render a neutral solution of methæmoglobin, such as that used in the last experiment (4), slightly alkaline with a few drops of ammonia. The solution becomes redder in color, due to the formation of alkaline methæmoglobin and shows a spectrum different from that of the neutral body. In this case we have a band on either side of D, the one nearer the red end of the spectrum being much the fainter. A third band, darker than either of those mentioned, lies between D and E somewhat nearer E.

6. **Alkali Hæmatin.**—Observe the spectrum of the alkali hæmatin prepared in Experiment 16 on page 212. Also make a spectroscopic examination of a freshly prepared alkali hæmatin.<sup>3</sup> The typical spectrum of alkali hæmatin shows a single absorption-band lying across D and mainly toward the red end of the spectrum.

<sup>1</sup> This transforms the oxyhæmoglobin into methæmoglobin.

<sup>2</sup> This is done to free the blood from carbon monoxide hæmoglobin.

<sup>3</sup> Alkali hæmatin may be prepared by mixing one volume of a concentrated potassium hydroxide or sodium hydroxide solution and two volumes of dilute (1:5) defibrinated blood. This mixture should be heated gradually almost to boiling, then cooled and shaken for a few moments in the air before examination.

7. **Reduced Alkali Hæmatin or Hæmochromogen.**—Dilute the alkali hæmatin solution used in the last experiment (6) to such an extent that it shows no absorption band. Now add a few drops of Stokes' reagent and note that the greenish-brown color of the alkali hæmatin solution is displaced by a bright red color. This is due to the formation of hæmochromogen or reduced alkali hæmatin. Examine this solution spectroscopically and observe the narrow, dark absorption-band lying midway between D and E. If the dilution is not too great a faint band may be observed in the green extending across E and b.

8. **Acid Hæmatin.**—To some defibrinated blood add half its volume of glacial acetic acid and an equal volume of ether. Mix thoroughly. The acidified ethereal solution of hæmatin rises to the top and may be poured off and used for the spectroscopic examination. If desired it may be diluted with acidified ether in the ratio of one part of glacial acetic acid to two parts of ether. A distinct absorption-band will be noted in the red between C and D and lying somewhat nearer C than the band in the methæmoglobin spectrum. Between D and F may be seen a rather indistinct broad band. Dilute the solution until this band resolves itself into two bands. Of these the more prominent is a broad, dark absorption-band lying in the green between b and F. The second, a narrow band of faint outline, lies in the light green to the red side of E. A fourth very faint band may be observed lying on the violet side of D.

9. **Acid Hæmatoporphyrin.**—To 5 c.c. of concentrated sulphuric acid in a test-tube add two drops of blood, mixing thoroughly by agitation after the addition of each drop. A wine-red solution is produced. Examine this solution spectroscopically. Acid hæmatoporphyrin gives a spectrum with an absorption-band on either side of D, the one nearer the red end of the spectrum being the narrower.

10. **Alkaline Hæmatoporphyrin.**—Introduce the acid hæmatoporphyrin solution just examined into an excess of distilled water. Cool the solution and add potassium hydroxide slowly until the reaction is but slightly acid. A colored precipitate forms which includes the principal portion of the hæmatoporphyrin. The presence of sodium acetate facilitates the formation of this precipitate. Filter off the precipitate and dissolve it in a small amount of dilute potassium hydroxide. Alkaline hæmatoporphyrin prepared in this way forms a bright red solution and possesses four absorption-bands. The first is a very faint, narrow band in the red, midway between C and D; the second is a broader, darker band lying across D, principally to the violet side. The third absorption-band lies principally between D and E, extending for a short distance across E to the violet side, and the fourth band is broad and dark and lies

between b and F. The first band mentioned is the faintest of the four and is the first to disappear when the solution is diluted.

#### VII. Instruments Used in the Clinical Examination of the Blood.

1. **Fleischl's Hæmometer** (Fig. 65, below).—This is an instrument used quite extensively clinically, for the quantitative determination of hæmoglobin. The instrument consists of a small cylinder which is provided with a fixed glass bottom and a movable glass cover, and which is divided, by means of a metal septum, into two compartments of equal capacity. This cylinder is supported in a vertical position by means of a

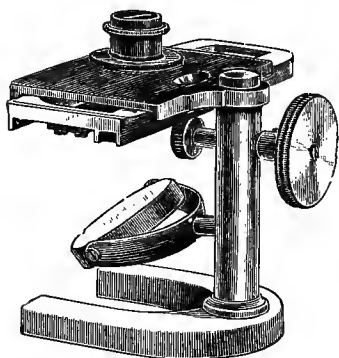


FIG. 65.—FLEISCHL'S HÆMOMETER.  
(*Da Costa.*)

mechanism which resembles the base and stage of an ordinary microscope. Underneath the stage is placed a colored glass wedge (see Fig. 67, p. 221), so arranged as to run immediately beneath the glass bottom of one of the compartments of the cylinder and ground in such a manner that each part of the wedge corresponds in color to a solution of hæmoglobin of some definite percentage. The glass wedge is held in a metal frame and may be moved backward or forward by means of a rack and pinion arrangement. A

scale along the side of this frame indicates the percentage of the normal amount of hæmoglobin which each particular variation in the depth of color of the ground wedge represents, taking the normal hæmoglobin content as 100.<sup>1</sup> In a position corresponding to the position of the mirror on the ordinary microscope is attached a light-colored opaque plate which serves to reflect the light upward through the colored wedge and the cylinder to the eye of the observer.

In making a determination of the percentage of hæmoglobin by this instrument the procedure is as follows: Fill each compartment about three-fourths full of distilled water. Puncture the finger-tip or lobe of the ear of the subject by means of a sterile needle or scalpel and, as soon as a drop of blood appears, place one end of the capillary pipette (Fig. 66), which accompanies the instrument, against the drop and allow it to fill by capillary attraction. To prevent the blood from adhering to the exterior of the tube, and so render the determination inaccurate, it is customary to apply a very thin coating of mutton fat to the outer surface before using or to wrap the tube in a piece of oily chamois when not in use. As soon as the tube has been accurately filled with blood it should

<sup>1</sup> The scale of the ordinary instrument is usually too high.

be dipped into the water of one of the compartments of the cylinder and all traces of the blood washed out with water by means of a small dropper which accompanies the instrument. If the blood is not well distributed throughout the compartment and does not form a homogeneous solution the contents of the compartment should be mixed thoroughly by means of the metal handle of the capillary measuring pipette. When this has been done each compartment should be completely filled with distilled water and the glass cover adjusted, care being taken that the contents of the two compartments do not mix. Now adjust the cylinder so that the compartment containing the pure distilled water is immediately above the colored glass wedge. By means of the rack and pinion arrangement manipulate the colored wedge until a portion of it is found which corresponds in color with the diluted blood. When this agreement in color has been secured the point on the scale corresponding to this particular color should be read and the actual percentage of hæmoglobin computed. For instance, if the scale reading is 90 it means that the blood under examination contains 90 per cent of the normal quantity of hæmoglobin, *i. e.*, 90 per cent of 14 per cent.

2. **Fleischl-Miescher Hæmometer.**—The apparatus of Fleischl has recently been modified by Miescher. If all precautions are taken, the margin of error in the absolute quantity of hæmoglobin determined by this instrument does not exceed 0.15–0.22 per cent by weight of the blood. Detailed directions for the manipulation of the Fleischl-Miescher hæmometer accompany the instrument. In brief Miescher modified the instrument as follows: (1) The scale of each instrument is supplied with

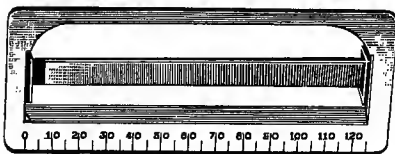


FIG. 67.—COLORED GLASS WEDGE OF FLEISCHL'S HÆMOMETER. (*Da Costa.*)

a caliber table of *absolute* hæmoglobin values, expressed in milligrams: the scale of Fleischl's hæmometer shows the percentage of hæmoglobin in relation to an average selected somewhat arbitrarily. Thus many errors arising from the irregular coloring of the glass wedge of the older apparatus are avoided in the instrument as modified. (2) Each instrument is accompanied by a measuring pipette (*melangeur*) which allows of a more accurate measurement of the blood than was possible with the capillary tubes of the older apparatus. (3) With the aid of the measuring pipette mentioned above blood of varying degrees of concentration may be compared. In this way the individual examinations are controlled and a check upon the accuracy of the gradu-



FIG. 66.—PIPETTE OF FLEISCHL'S HÆMOMETER.

ation in the color of the glass wedge is also afforded. This wedge is much more evenly and accurately colored than in the unmodified apparatus of Fleischl. (4) Before reading the percentage as indicated by the scale, the chamber is covered with a glass and a diaphragm which sharply define the field on all sides without the formation of a meniscus.

The measuring pipette is constructed essentially the same as the pipettes which accompany the Thoma-Zeiss apparatus (see page 225).

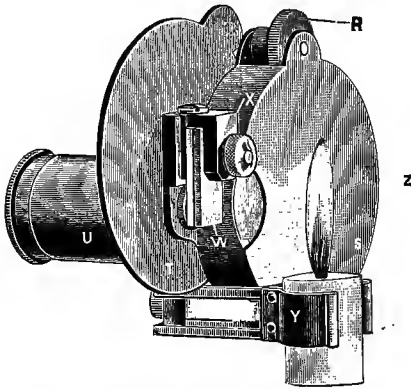


FIG. 68.—DARE'S HÆMOGLOBINOMETER.  
(Da Costa.)

R, Milled wheel acting by a friction bearing on the rim of the color disc; S, case inclosing color disc, and provided with a stage to which the blood chamber is fitted; T, movable wing which is swung outward during the observation, to serve as a screen for the observer's eyes, and which acts as a cover to inclose the color disc when the instrument is not in use; U, telescoping camera tube, in position for examination; V, aperture admitting light for illumination of the color disc; X, capillary blood chamber adjusted to stage of instrument, the slip of opaque glass, W, being nearest to the source of light; Y, detachable candle-holder; Z, rectangular slot through which the hæmoglobin scale indicated on the rim of the color disc is read.

The capillary portion, however, is graduated, 1,  $\frac{2}{3}$  and  $\frac{1}{2}$  which enables the observer to dilute the blood sample in the proportion of 1:200, 1:300 or 1:400 as he may desire. If there is difficulty in drawing in the blood exactly to one of the graduations just mentioned the amount of blood above or below the volume indicated by the graduation may be determined by means of certain delicate cross-lines which are placed directly above and below the graduation. Each cross-line corresponds to  $\frac{1}{100}$  of the volume of the capillary tube from the tip to the 1 graduation.

A 0.1 per cent solution of sodium carbonate is used to dissolve the stroma of the erythrocytes and so render the blood solution perfectly clear. If this is not done the color of the blood solution invariably appears darker in tone than that of the colored glass wedge. A freshly

prepared sodium carbonate solution should be used in order that the clearness of the solution may not be marred by the presence of sodium bicarbonate.

3. **Dare's Hæmoglobinometer** (Fig. 68).—This instrument, as the name signifies, is used for the determination of hæmoglobin. In using either Fleischl's hæmometer or the instrument as modified by Miescher the blood is diluted for examination, whereas with the Dare instrument *no dilution* is required. This probably allows of rather more accurate determinations than are possible with the old Fleischl apparatus.

The instrument consists essentially of the following parts: (1) A capillary observation cell, (2) a semicircular colored glass wedge, (3) a milled wheel for manipulating the wedge, (4) a candle used to illuminate portions of the capillary observation cell and the colored wedge, (5) a small telescope used in the examination of the areas illuminated by the candle flame, (6) a scale graduated in percentages of the normal amount of hæmoglobin, (7) a hard-rubber case, (8) a movable screen attached to the case.

The capillary observation cell is formed of two small, polished rectangular plates of glass, one being transparent and the other opaque. When held in position on the instrument, by means of a small metal bracket, the opaque portion of the cell is nearer the candle and thus serves to soften the glare of light when an observation is being made. The transparent portion of the cell is directly over a circular opening in the case, through which the blood specimen is viewed by means of the small telescope.

The semicircular colored glass wedge is so ground that each particular shade of color corresponds to that possessed by fresh blood which contains some definite percentage of hæmoglobin. It is mounted upon a disc which may be manipulated by the milled wheel in such a manner as to bring successive portions of the wedge in position to be viewed through a circular opening contiguous to the opening through which the

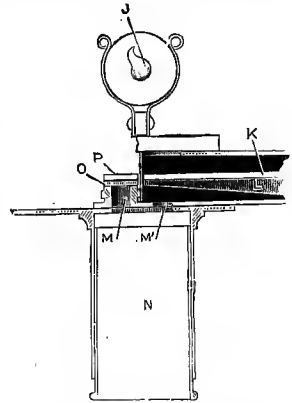


FIG. 69.—HORIZONTAL SECTION OF DARE'S HÆMOGLOBINOMETER. (*Da Costa.*)

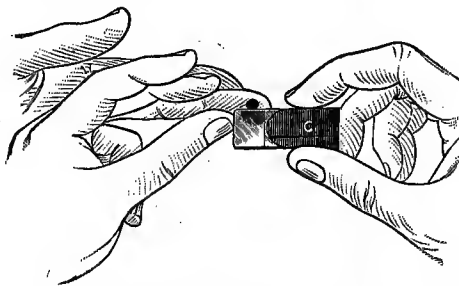


FIG. 70.—METHOD OF FILLING THE CAPILLARY OBSERVATION CELL OF DARE'S HÆMOGLOBINOMETER. (*Da Costa.*)

blood specimen is viewed. For a further description of the instrument see Figs. 68, 69, and 70.

In using the Dare hæmoglobinometer proceed as follows: Puncture the finger-tip or lobe of the ear of the subject by means of a needle or scalpel and, after a drop of blood of good proportions has formed, place

the flat capillary observation cell in contact with the drop and allow it to fill by capillary attraction (Fig. 70). Replace the cell in its proper place on the instrument. When in position, a portion of this cell may be observed through a small telescope attached to the apparatus. It is viewed through a circular opening and near this circle is a second one through which a portion of a semicircular colored glass wedge is visible. These two circles are illuminated simultaneously by means of the flame of a candle. The colored glass may be rotated by means of a milled wheel and the point of agreement of the color of the adjoining discs may be determined in the same way as in Fleischl's hæmometer. The scale reading gives the percentage of the normal quantity of hæmoglobin which the blood sample under examination contains. Compute the actual hæmoglobin content in the same manner as from the scale reading of the Fleischl hæmometer (see page 221).

4. **Tallquist's Hæmoglobin Scale.**—This consists essentially of a series of ten colors corresponding to stains produced by blood containing varying percentages of hæmoglobin. In using this scale a drop of blood is allowed to fall on a small section of filter paper and the resulting color is compared with the ten colors of the scale. When the color in the scale is found which corresponds to the color of the blood stain the accompanying hæmoglobin value is read off directly. This is a very convenient method for determining hæmoglobin at the bedside. There is a possibility of the colors being inaccurately printed, however, and even if originally correct in tint, under the continued influence of air and light they must eventually alter somewhat.

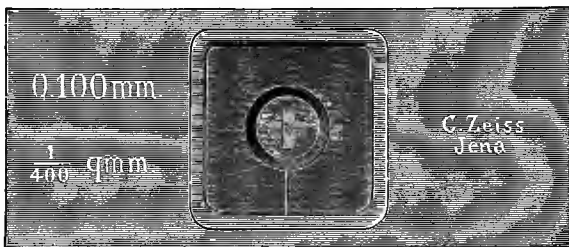


FIG. 71.—THOMA-ZEISS COUNTING CHAMBER. (*Da Costa.*)

5. **Thoma-Zeiss Hæmocytometer.**—This is an instrument used in "blood counting," *i. e.*, in determining the number of erythrocytes and leucocytes. The instrument consists of a microscopic slide constructed of heavy glass and provided with a central counting cell (see Fig. 71, below). This cell, with the cover glass in position, is exactly 0.1 millimeter deep. The floor of the cell is divided by delicate lines into



squares each of which is  $\frac{1}{400}$  of a square millimeter in area (see Fig. 73, page 226). The volume of blood therefore between any particular square and the cover glass above must be  $\frac{1}{4000}$  cubic millimeter. Accompanying each instrument are two capillary pipettes (Fig. 72, below), each constructed with a mixing bulb in its upper portion. Each bulb is further provided with an enclosed glass bead which is of great assistance in mixing the contents of the chamber. The stem of each pipette is graduated in tenths from the tip to the bulb. The final graduation at the upper end of the bulb is 101 on the pipette used in mixing the blood sample in which the erythrocytes are counted (erythrocytometer, see Fig. 72, page 225), and 11 on the pipette used in mixing the blood sample for the leucocyte count (leucocytometer, see Fig. 72, page 225). In making "blood counts" with the hæmocytometer it is necessary to use some diluting fluid. Two very satisfactory forms of fluid for this purpose are Toison's and Sherrington's solutions.<sup>1</sup> When either of these solutions is used as the diluting fluid it is possible to make a very satisfactory count of both the erythrocytes and leucocytes from the same preparation, since the leucocytes are stained by the methyl-violet or methylene-blue.

In counting the erythrocytes by means of the hæmocytometer, proceed as follows: Thoroughly cleanse the tip of the finger or lobe of the ear of the subject by the use of soap and water, alcohol and ether applied in the sequence just given. Puncture the skin by means of a needle or scalpel and allow the blood drop to form without pressure. Place the tip of the pipette in contact with the blood drop, being careful to avoid touching the skin, and draw blood into the pipette up to the point marked 0.5 or 1 according to the desired dilution. Rapidly wipe the tip of the pipette and immediately fill it to the point marked 101 with Toison's or Sherrington's solution. Now thoroughly mix the blood and diluting fluid within the mixing chamber by tapping the pipette gently against the finger, or by

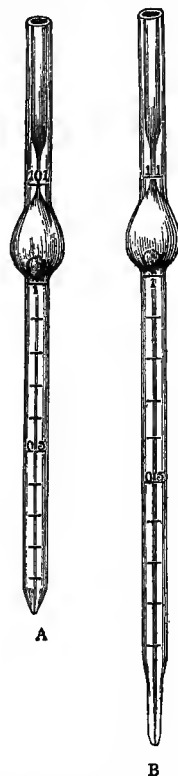


FIG. 72.—THOMAZEISS CAPILLARY PIPETTES.

A, Erythrocytometer; B, Leucocytometer.

<sup>1</sup> Toison's solution has the following formula:

Methyl-violet.....	0.025 gram.
Sodium chloride.....	1 gram.
Sodium sulphate.....	8 grams.
Glycerol.....	30 grams.
Distilled water.....	160 grams.

Sherrington's solution has the following formula:

Methylene-blue.....	0.1 gram.
Sodium chloride.....	1.2 gram.
Neutral potassium oxalate....	1.2 gram.
Distilled water.....	300.0 grams.

shaking it while held securely with the thumb at one end and the middle finger at the other. After the two fluids have been thoroughly mixed the diluting fluid contained in the capillary-tube below the bulb should be discarded in order to insure the collection of a drop of the thoroughly mixed blood and diluting solution for examination. Transfer a drop from the pipette to the ruled floor of the counting chamber and, after placing the cover glass firmly in position,<sup>1</sup> allow an interval of a few minutes to elapse for the corpuscles to settle before making the count. Now place the slide under the microscope and count the number of erythrocytes in a number of squares, counting the corpuscles which are in contact

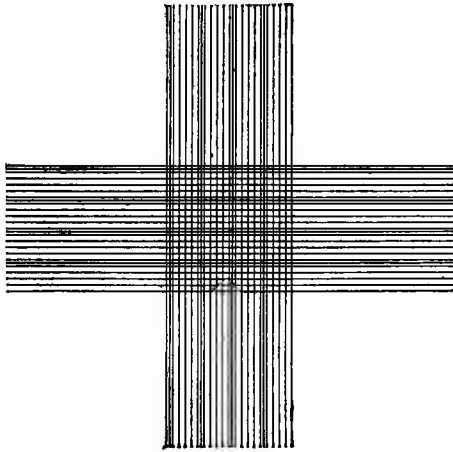


FIG. 73.—ORDINARY RULING OF THOMA-ZEISS COUNTING CHAMBER. (*Da Costa.*)

with the upper and the right-hand boundaries of the square as belonging to that square. Take the squares in some definite sequence in order that the recounting of the same corpuscles may be avoided. A satisfactory procedure is to begin in the upper right-hand corner and proceed from left to right counting the cells in each individual square. Take the next lower row of squares and count from left to right and so on (see Fig. 77, p. 232). Of course, all things being equal, the greater the number of squares examined the more accurate the count. It is considered essential under all circumstances, where an accurate count is desired, that the counting chamber shall be filled, at least twice, and the individual counts made in each instance, as indicated above, before the data are deemed satisfactory. Under no conditions should less than 200 squares be examined.

To calculate the number of erythrocytes per cubic millimeter of undiluted blood proceed as follows: Determine the number of corpuscles

<sup>1</sup> If the cover glass is in accurate apposition to the counting cell Newton's rings may be plainly observed.

in any given number of squares and divide this total by the number of squares, thus obtaining the average number of erythrocytes per square. Multiply this average by 4,000 to obtain the number of erythrocytes per cubic millimeter of *diluted* blood, and multiply this product by 100 or 200, according to the dilution, to obtain the number of erythrocytes per cubic millimeter of *undiluted* blood. Thus:

$$\text{Average number of erythrocytes per square} \times 4,000 \times 200 \text{ (or } 100) = \text{Number of erythrocytes per cubic millimeter.}$$

Great care should be taken to see that the capillary pipette is properly cleaned. After using, it should be immediately rinsed out with the

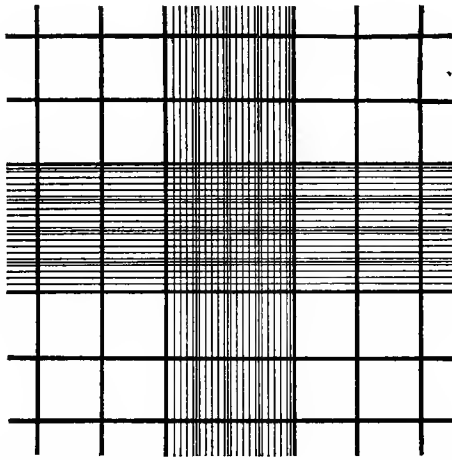


FIG. 74.—ZAPPERT'S MODIFIED RULING OF THOMA-ZEISS COUNTING CHAMBER. (*Da Costa*.)

diluting fluid, then with water, alcohol, and ether in the sequence given. Finally dry air should be drawn through the capillary and a horse hair inserted to prevent the entrance of dust particles.

In counting leucocytes by means of the hæmocytometer proceed as follows: As mentioned above, if the diluting fluid is either Toison's or Sherrington's solution the leucocytes may be counted in the same specimen of blood in which the erythrocytes are counted. When this is done it is customary to use a slide provided with Zappert's modified ruling (Fig. 74, above). This method is rather more accurate than the older one of counting the leucocytes in a separate specimen of blood. Furthermore, it is obviously preferable to count both the erythrocytes and the leucocytes from the same blood sample. To insure accuracy the number of leucocytes within the *whole* ruled region should be determined in *duplicate* blood samples. This includes the examination of an area eighteen times as great as the old style Thoma-Zeiss central ruling. This

region then would correspond to 3,600 of the small squares and, if *duplicate* examinations were made, the total number of small squares examined would aggregate 7,200. The calculation would be as follows:

$$\frac{\text{Number of leucocytes in } 7,200 \text{ squares}}{\times 200 \times 4,000 \div 7,200} = \frac{\text{Number of leucocytes per cubic millimeter.}}{\text{millimeter.}}$$

If a Zappert slide is not available, a good plan to follow is to place a diaphragm in the tube of the ocular of the microscope consisting of a circle of black cardboard or metal<sup>1</sup> having a square hole in the center of such a size as to allow of the examination of exactly 100 squares or one-fourth of a square millimeter at one time. With this arrangement any portion of the specimen may be examined and counted whether within or without the ruled area. In counting by means of this device it is, of course, helpful if the microscope is provided with a mechanical stage, but even without this arrangement, if the observer is careful to see that the leucocytes at the extreme boundary of one field move to the opposite boundary when the position of the slide is changed, the device may be very satisfactorily employed. The leucocytes should be counted in 36 of the diaphragm-fields in *duplicate* specimens and the calculation made in the same manner as explained above.

If the leucocytes are counted in a separate specimen of blood ordinarily the diluting fluid is 0.3–0.5 per cent acetic acid, a fluid in which the leucocytes alone remain visible. Under these conditions the dilution is customarily made in the pipette having 11 as the final graduation. The capillary portion is of larger caliber and so requires a greater amount of blood to fill it to the 0.5 or 1 mark than is required in the use of the other form of pipette. In counting the leucocytes according to this method it is customary to draw blood into the pipette up to the 1 mark and immediately fill the remaining portion of the apparatus to the 11 graduation with the 0.3–0.5 per cent acetic acid. It then remains to count the number of leucocytes in the whole central ruled portion of 400 squares. This should be done in *duplicate* samples and the calculation made as follows:

$$\frac{\text{Number of leucocytes in } 800 \text{ squares}}{\times 4,000 \times 10 \div 800} = \frac{\text{Number of leucocytes per cubic millimeter.}}{\text{millimeter.}}$$

6. **Bürker's Hæmocytometer.**<sup>2</sup>—This is an improved apparatus<sup>3</sup> for the more accurate counting of erythrocytes than is possible by the Thoma-Zeiss apparatus. The principles involved are somewhat different from those in force with the latter apparatus. For example, the blood is diluted in a separate vessel, not in the pipette with which the sample is drawn, and furthermore the cover glass is applied to the counting

<sup>1</sup> Ehrlich's mechanical eye-piece with iris diaphragm is also very satisfactory for this purpose.

<sup>2</sup> Bürker: *Pflüger's Archiv.*, 142, 337, 1911; *Münch. med. Woch.*, 59, pp. 14 and 89, 1912.

<sup>3</sup> Manufactured by C. Zeiss, Jena.

chamber and *clamped* in place *before* the diluted blood is applied to the ruled area. Hayem's solution<sup>1</sup> is used as the diluting fluid. Toison's solution is not satisfactory for use with the Bürker counting chamber as its viscosity is too great. The corpuscles settle rapidly in Hayem's fluid as the specific gravity of the fluid is 1015 whereas that of the erythrocytes is 1090.

The pipette for measuring the quantity of blood (Fig. 75, upper pipette) has a point which is not ground dull but is polished. This

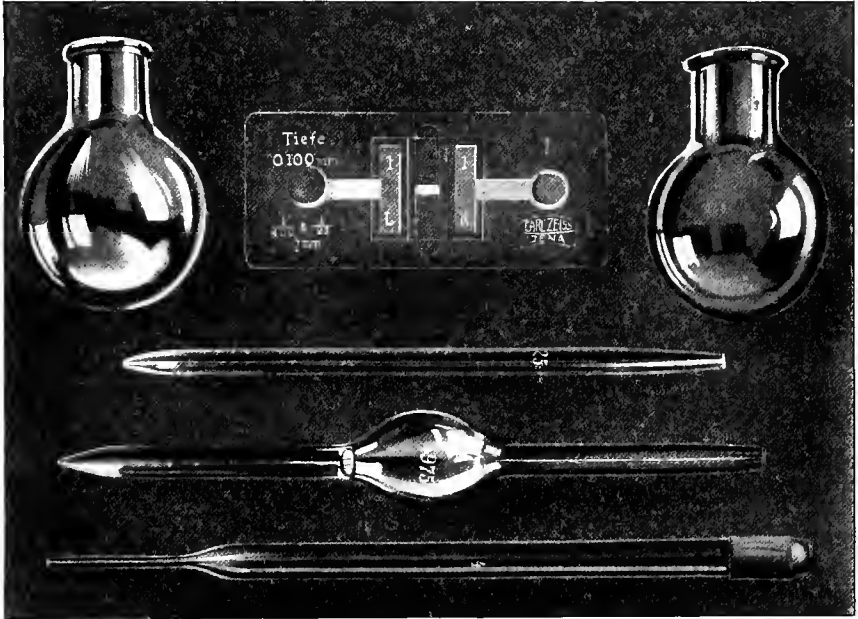


FIG. 75.—BÜRKER'S PIPETTES, MIXING FLASKS AND COUNTING CHAMBER.

allows of better judgment in deciding whether the column of blood extends to the very tip. The volume of the pipette between tip and mark is 25 cubic millimeters. The mark extends all the way around the tube so that errors of parallax may be avoided.

The pipette for measuring the diluting fluid (Fig. 75, middle pipette) also has a polished point and circular mark and delivers 4975 cubic millimeters. This volume of diluting fluid with 25 cubic millimeters of blood gives a dilution of 1:200. Both pipettes are provided with a piece of rubber tubing and mouth-piece.

<sup>1</sup> Hayem's solution has the following formula:

Mercuric chloride.....	0.25 gram.
Sodium chloride.....	0.5 gram.
Sodium sulphate.....	2.5 grams.
Distilled water.....	100.0 grams.

For transferring the diluted blood from the diluting flask to the chamber a plain pipette provided with a rubber cap is used (Fig. 75, lower pipette). It is filled by pressing the cap slowly with the index finger, inserting the tip into the liquid and then releasing the pressure.

The diluting is done in a small round-bottomed flask as shown in Fig. 75. Several of these flasks should be kept on hand in a wooden rack which will hold them in an upright position. Each flask is provided with a paraffined, or smooth cork stopper.

In the older counting chambers the floor of the chamber is circular and the counting is done in the center of this space. The corpuscles are therefore counted in the center of a capillary, circular film where on account of surface tension their number is slightly greater than elsewhere. This source of error is avoided in the new counting chamber (Fig. 78) in which the floor is represented by the upper surface of a piece of glass 25 mm. long and 5 mm. wide which is rounded off at both ends and divided into two portions by a groove 1.5 mm. wide through the center. At each side of this floor piece, separated from it by a groove is a glass plate (5 mm.  $\times$  21 mm.) of such height that the space between the floor of the cell and a cover glass placed across the plates is 0.100 mm. A cover glass 23 mm. long and 21 mm. wide with rounded polished edges is used so that the rounded ends of the floor piece project beyond it. The chamber is provided with clamps to press the cover glass firmly upon both plates (Fig. 75).

The ruling on each portion of the floor piece is that shown in Fig. 76, which will be explained below.

*Measuring the Diluting Fluid.*—Four thousand nine hundred and ninety-five cubic millimeters of diluting fluid (*Hayem's*) are measured out into the diluting flask. To do this the pipette is filled by suction to slightly above the mark and the rubber tube is carefully clamped off. Then with a soft piece of linen the tip is wiped dry. The meniscus is then accurately adjusted to the mark by lightly touching the point of the pipette to the cleaned tip of the finger. The pipette is then inserted into the diluting flask and with the tip nearly touching the bottom of the flask the fluid is allowed to run out. The time of the flow should be about forty seconds and is controlled by placing the tip of the index finger closely upon the mouth piece. The pipette is emptied completely by alternately blowing through it and touching it to the wall of the flask slightly above the level of the liquid. The drops clinging to the wall are wiped with the bulk of the liquid by a suitable motion of the flask. The flask is then stoppered, care being taken from now on that none of the fluid ever touches the neck of the flask or the stopper.

*Taking the Blood Sample.*—Usually the best time to draw the blood is

before breakfast. For a single determination the author prefers to draw it from the tip of the fourth finger of the left hand. For repeated determinations it is well to change off between third, fourth and fifth fingers of left hand. The temperature of the room should not be below  $17^{\circ}$  C. to prevent an undue contraction of the cutaneous vessels. The instrument used to puncture the finger should have a chisel-shaped point which is preferable to the ordinary lancet-shaped point. The first drop of blood is wiped off. Into the second one the tip of the pipette is inserted and blood is drawn in until the meniscus is even with or a little beyond the mark. The tip is then wiped off without touching the capillary opening and the observer assures himself that the column of blood extends to the very end of the capillary. The meniscus is then accurately adjusted to the mark.

*Mixing of the Blood and Diluting Fluids.*—The tip of the pipette is now dipped into the diluting fluid which has been measured into the flask and the blood is slowly blown out. The blood having a much higher specific gravity than the Hayem's fluid sinks to the bottom. The pipette is then filled with the pure supernatant diluting fluid and emptied again, care being taken to avoid air bubbles. This is repeated until the blood is removed as completely as possible. To mix the blood and diluting fluid the flask is rotated for two minutes in spiral curves of continually decreasing radius. The motion should be alternately clockwise and counter-clockwise. After complete mixing the pipette is rinsed out several times with the diluted blood.

*Transferral of the Diluted Blood to the Chamber.*—The counting chamber which has been cleaned with distilled water and alcohol-ether and then wiped dry with a soft cloth as free from lint as possible is placed upon a black surface and carefully brushed with a camel's hair brush. The cover glass is now placed over the chamber by sliding it over the two glass plates with both thumbs while the index fingers are pressing it down. By means of the clamps it is held in place firmly so that Newton's rings (if possible of the first order: brown and black) may be seen over the entire area of the plates. The chamber is placed upon the stage of the microscope and is brought into a horizontal position.

Before transferring the diluted blood to the chamber the flask must be shaken for two minutes as described before. The liquid shows a cloudy appearance and must be allowed to stand until the turbidity has become uniform.

One of the plain pipettes described above is now inserted into the diluted blood while slight pressure is being exerted on the rubber cap. The pressure is released slowly and the liquid rises into the pipette. The point of the pipette is now immediately placed upon one of the projecting

ids of the floor plate and very slight pressure is exerted on the rubber  
p until the liquid coming from the pipette just reaches the cover glass  
hen the pressure is released. An instantaneous filling of the capillary

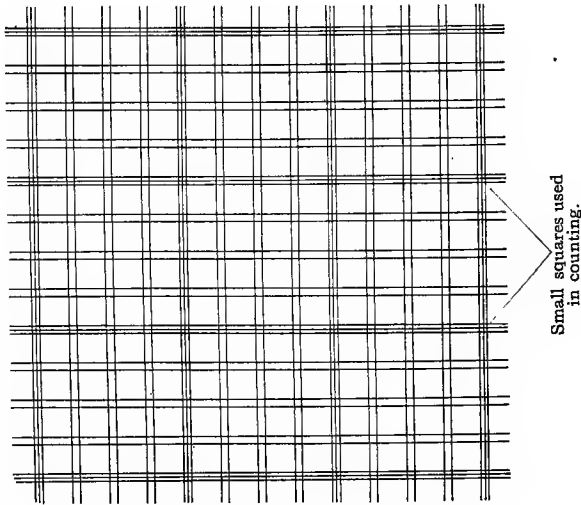


FIG. 76.—RULING OF BÜRGER COUNTING CHAMBER.

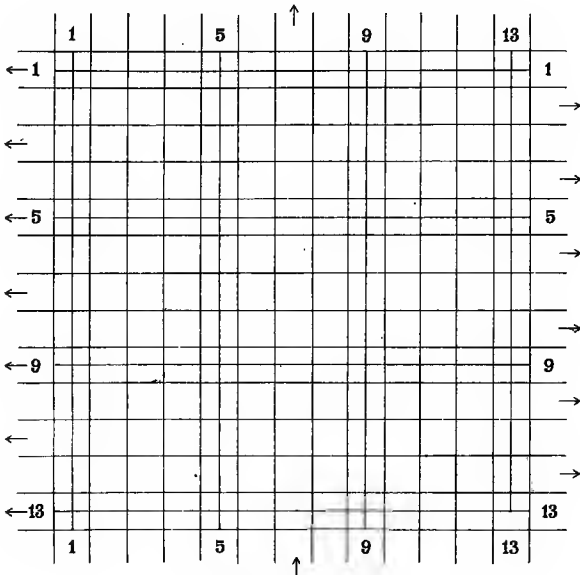


FIG. 77.—SCHEMA.

pace results. The pipette should be emptied immediately, rinsed with  
distilled water and placed in an upright position in a beaker of water.  
The other portion of the counting chamber is now filled in the same way



with a second pipette and about one minute is allowed for the settling of the corpuscles. During this time the pipettes may be washed with distilled water and ether-alcohol and dried by suction. Occasionally, the pipettes should be cleaned with a horse hair and with concentrated  $H_2SO_4$  containing a little  $K_2Cr_2O_7$ .

To see whether the distribution of the corpuscles has been uniform the chamber is illuminated with a wide-open diaphragm and viewed at an angle. If the opacity is not uniform in either of the portions of the chamber, that one should not be used for counting. If the counting must be interrupted or requires a long time a moist chamber<sup>1</sup> should be used to prevent evaporation of the diluting fluid. The diluted blood may be retained in the mixing flasks and duplicate countings obtained after the lapse of twenty-four hours or more according to Bürker.

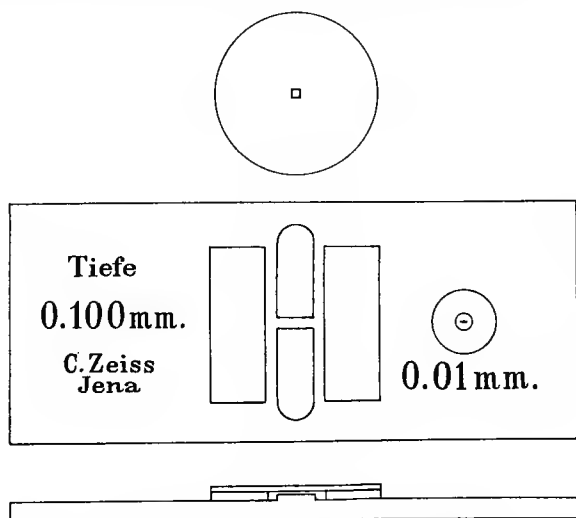


FIG. 78.—BÜRKER COUNTING CHAMBER.

*Counting and Calculation.*—A mechanical stage movable in two directions is indispensable. With a magnification of 320 diameters the counting is begun in the left upper corner of the ruling. Proceed from left to right along one row then move from right to left along the next lower row, and so on. Only the small squares are used for counting (see Fig. 76), and the figures are recorded in the *schema*<sup>2</sup> (see Fig. 77) in which the squares crossed by horizontal or vertical lines correspond to the small squares used for counting. Usually 80 squares are counted and by recording the figures in the *schema* the count may be verified and an idea

<sup>1</sup> Bürker: *Pflüger's Archiv*, 118, 465, 1907.

<sup>2</sup> The firm of H. Laupp in Tübingen has put this schema on the market (in packs of 100).

of the uniformity of the distribution may be formed. Half of the counted squares should be in the one, half in the other portion of the counting chamber. For more accurate measurements more squares may be counted.

The observer will do well not to attempt counting each individual corpuscle in a square. After some practice each typical group of corpuscles will immediately suggest a number. A very common form of grouping is one corpuscle surrounded by four others. This should immediately suggest the number five. In this way the counting will become more rapid and also more reliable.

The calculation is very simple. The number of corpuscles in 80 squares divided by 100 will give the number of millions per cubic millimeter. If, for example, 536 corpuscles have been counted in 80 squares then with a dilution of 1:200 the number of corpuscles per cubic millimeter is 5,360,000. Thus,  $\frac{536}{80} \times 4,000 \times 200 = 5,360,000$  erythrocytes per cubic millimeter. More than two decimal places are without significance.

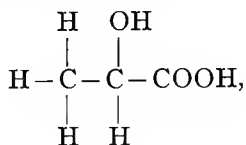
## CHAPTER XIII.

### MILK.

Milk is the most satisfactory individual food material elaborated by nature. It contains the three nutrients, protein, fat, and carbohydrate and inorganic salts in such proportion as to render it a very acceptable dietary constituent. It is a specific product of the secretory activity of the mammary gland. It contains, as the principal solids, *olein*, *palmitin*, *stearin*, *butyryn*, *caseinogen*, *lact-albumin*, *lacto-globulin*, *lactose*, and *calcium phosphate*. It also contains at least traces of lecithin, cholesterol, urea, creatine, creatinine, and the tri-glycerides of caproic, lauric, and myristic acids. Citric acid is also said to be present in milk in minute quantity. Considered from the standpoint of colloid chemistry we may classify the main constituents of milk as follows:<sup>1</sup>

In suspension	<i>Fat</i> (olein, palmitin, etc.).
In colloidal solution	{ <i>Caseinogen</i> —an unstable or irreversible colloid.
	{ <i>Lact-albumin</i> —a stable or reversible colloid.
In crystalloid solution	{ <i>Salts</i> (calcium phosphate, etc.).
	{ <i>Sugar</i> (lactose).

Fresh milk is amphoteric in reaction to litmus,<sup>2</sup> but upon standing for a sufficiently long time, unsterilized, it becomes acid in reaction, due to the production of fermentation lactic acid,



from the lactose contained in it. This is brought about through bacterial activity. The white color is imparted to the milk partly through the fine emulsion of the fat and partly through the medium of the caseinogen in solution. The specific gravity of milk varies somewhat, the average being about 1.030. Its freezing-point is about  $-0.56^{\circ}$  C.

Fresh milk does not coagulate on being boiled but a film consisting of a combination of caseinogen forms on the surface. If the film be

<sup>1</sup> Alexander and Bullowa: *Jour. Am. Med. Ass'n.*, 55, 1196, 1910.

<sup>2</sup> Human milk as well as cow's milk. It is, however, acid to phenolphthalein.

removed, thus allowing a fresh surface to come in contact with the air, a new film will form indefinitely upon the application of heat. Surface evaporation and the presence of fat facilitate the formation of the film, but are not essential (Rettger<sup>1</sup>). As Jamison and Hertz<sup>2</sup> have shown, a similar film will form on heating any protein solution containing fat or paraffin. If the milk is acid in reaction, through the inception of lactic acid fermentation, or from any other cause, no film will form when heat is applied, but instead a true coagulation will occur. When milk is boiled certain changes occur in its odor and taste. These changes, according to Rettger,<sup>3</sup> are due to a partial decomposition of the milk proteins and are accompanied by the liberation of a volatile sulphide, probably hydrogen sulphide.



FIG. 79.—NORMAL MILK AND COLOSTRUM.  
a, Normal milk; b, Colostrum.

The milk-curdling enzymes of the gastric and the pancreatic juice have the power of splitting the caseinogen of the milk, through a process of hydrolysis, into *soluble casein* and a *peptone-like* body. This soluble casein then forms a combination with the calcium of the milk and an insoluble curd of *calcium casein* or *casein* results. The clear fluid surrounding the curd is known as *whey*.

There is still considerable confusion of terms when different authorities discuss milk proteins and the action of milk curdling enzymes upon them. The English-speaking scientists quite uniformly accept the classification of Halliburton<sup>4</sup> as given above. On the other hand, the Germans in particular give the name *casein* to the milk protein and *paracasein* to the

<sup>1</sup> Rettger: *American Journal of Physiology*, 7, 325, 1902.

<sup>2</sup> Jamison and Hertz: *Journal of Physiology*, 27, 26, 1902.

<sup>3</sup> Rettger: *American Journal of Physiology*, 6, 450, 1902.

<sup>4</sup> Halliburton: *Journal of Physiology*, 11, 448, 1900.

product of the action of rennin upon this protein. The confusion of terms may be represented thus:

<i>English.</i>		<i>German.</i>
Caseinogen.	=	Casein.
Casein.	=	Paracasein.

The most pronounced difference between human milk and cow's milk is in the protein content, although there are also differences in the fats and likewise striking biological differences difficult to define chemically. It has been shown that the caseinogen of human milk differs from the caseinogen of cow's milk in being more difficult to precipitate by acid or coagulate by gastric rennin. The casein curd also forms in a much looser and more flocculent manner than that from cow's milk and is for this reason much more easily digested than the latter. Interesting data relative to the composition of milk from various sources may be gathered from the following table which was compiled mainly from the results of investigations by Pröscher<sup>1</sup> and by Abderhalden<sup>2</sup> in Bunge's laboratory. It will be noted that the composition of the milk varies directly with the length of time needed for the young of the particular species to double in weight.

Species.	Period in which Weight of the New-born is Doubled (Days).	100 Parts of Milk Contain			
		Proteins.	Salts.	Calcium.	Phosphoric Acid.
Man.....	180	1.6	0.2	0.033	0.047
Horse.....	60	2.0	0.4	0.124	0.131
Cow.....	47	3.5	0.7	0.160	0.197
Goat.....	22	3.7	0.8	0.197	0.284
Sheep.....	15	4.9	0.8	0.245	0.293
Pig.....	14	5.2	0.8	0.249	0.308
Cat.....	9.5	7.0	1.0	.....	.....
Dog.....	9	7.4	1.3	0.455	0.508
Rabbit.....	6	10.4	2.5	0.891	0.997

The secretion of the mammary glands of the newborn of both sexes is called "witches' milk." The name is centuries old and evidently refers to the mystery of the useless secretion. Basch<sup>3</sup> has recently suggested that this secretion of "witches' milk" is brought about by the passage of *hormones* (see chapter on Pancreatic Digestion) from the blood of the mother to the fetus.

Lactose, the principal carbohydrate constituent of milk, is an impor-

<sup>1</sup> Pröscher: *Zeit. f. physiol. Chemie*, 24, 285, 1898.

<sup>2</sup> Abderhalden: *Ibid.*, 26, 487, 1899; and 27, pp. 408 and 457, 1899.

<sup>3</sup> Basch: *Münch. med. Woch.*, 58, 2266, 1911.

tant member of the disaccharide group. It occurs only in milk, except as it is found in the urine of women during pregnancy, during the nursing period, and soon after weaning; it also occurs in the urine of normal persons after the ingestion of a very large amount of lactose in the food. It is not derived directly from the blood, but is a specific product of the cellular activity of the mammary gland. It has strong reducing power, is dextro-rotatory, and forms an osazone with phenylhydrazine. The souring of milk is due to the formation of lactic acid from lactose through the agency of the *bacterium lactis*. Putrefactive bacteria in the alimentary canal may bring about this same reaction. Lactose is *not* fermentable by pure yeast. It was recently claimed that *lactosin*, a new carbohydrate, had been isolated from milk.

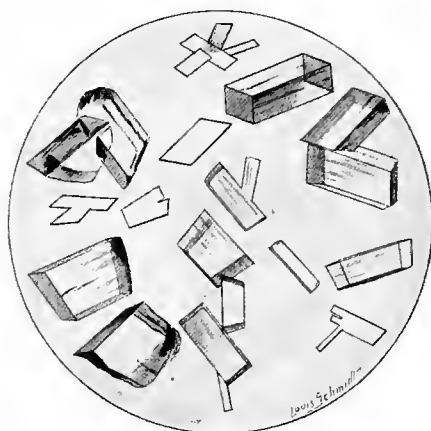


FIG. 80.—LACTOSE.

Caseinogen, the principal protein constituent of milk, belongs to the group of phosphoproteins. It has acidic properties and combines with bases to produce salts. It is not coagulable upon boiling and is precipitated from its neutral solution by certain metallic salts as well as upon saturation with sodium chloride or magnesium sulphate. Its acid solution is precipitated by an excess of mineral acid.

Lactalbumin and lacto-globulin, the protein constituents of milk, next in importance to caseinogen, closely resemble serum albumin and serum globulin in their general properties. According to Wroblewski, a protein called *opalisin* is also present in milk.

Butter (milk fat) consists in large part of *olein* and *palmitin*. Stearin, butyrin, caproin and traces of other fats are also present. When butter becomes rancid through the cleavage of certain of its constituent fats by bacteria the odors of caproic and butyric acids are in evidence.

Colostrum is the name given to the product of the mammary gland

secreted for a short time before parturition and during the early period of lactation (see Fig. 79, p. 236). It is yellowish in color, contains more solid matter than ordinary milk, and has a higher specific gravity (1.040–1.080). The most striking difference between colostrum and ordinary milk is the high percentage of lactalbumin and lacto-globulin in the former. This abnormality in the protein content is responsible for the coagulation of colostrum upon boiling.

Such enzymes as lipase, amylase, galactase, catalase, oxidases, peroxidases, and reductases have been identified in milk, but not all of them in milk of the same species of animal.

Among the principal preservatives used in connection with milk are formaldehyde, hydrogen peroxide, boric acid, borates, salicylic acid, and salicylates.

#### EXPERIMENTS ON MILK.

1. **Reaction.**—Test the reaction of fresh cow's milk to *litmus*, *phenolphthalein* and *congo red*.

2. **Biuret Test.**—Make the biuret test according to directions given on page 98.

3. **Microscopical Examination.**—Examine fresh *whole* milk, *skimmed* or *centrifugated* milk, and *colostrum* under the microscope. Compare the microscopical appearance with Fig. 79, page 236.

4. **Specific Gravity.**—Determine the specific gravity of both whole and skimmed milk (see p. 278). Which possesses the higher specific gravity? Explain why this is so.

5. **Film Formation.**—Place 10 c.c. of milk in a small beaker and boil a few minutes. Note the formation of a film. Remove the film and heat again. Does the film now form? Of what substance is this film composed? The biuret test was positive, why do we not get a coagulation here when we heat to boiling?

6. **Coagulation Test.**—Place about 5 c.c. of milk in a test-tube, acidify slightly with dilute acetic acid and heat to boiling. Do you get any coagulation? Why?

7. **Action of Hot Alkali.**—To a little milk in a test-tube add a few drops of potassium hydroxide and heat. A yellow color develops and gradually deepens into a brown. To what is the formation of this color due?

8. **Test for Chlorides.**—To about 5 c.c. of milk in a test-tube add a few drops of *very dilute* nitric acid to form a precipitate. Filter off this precipitate and test the filtrate for chlorides. Does milk contain any chlorides?

9. **Guaiac Test.**—To about 5 c.c. of water in a test-tube add 3 drops of milk and enough alcoholic solution of guaiaac (strength about 1:60)<sup>1</sup> to cause a turbidity. Thoroughly mix the fluids by shaking and observe any change which may gradually take place in the color of the mixture. If no blue color appears in a short time, heat the tube gently below 60° C. and observe whether the color reaction is hastened. In case a blue color does not appear in the course of a few minutes, add hydrogen peroxide or old turpentine, drop by drop, until the color is observed. Fresh milk will frequently give this blue color when treated with an alcoholic solution of guaiaac without the addition of hydrogen peroxide or old turpentine. See discussion on page 204.

10. **Tests to Differentiate Between Raw Milk and Heated Milk.**—

(a) *Kastle's Peroxidase Reaction.*—The peroxidase reaction of milk is founded upon the fact that small amounts of *raw* milk will induce the oxidation of various leuco compounds by hydrogen peroxide. This reaction has been used in a practical way as the most convenient means of differentiating between *raw milk* and *heated milk*. Many substances have been employed for this purpose, *e. g.*, guaiaac, paraphenylenediamine, ortol, amidol, etc. Kastle has found that a dilute solution of "trikresol"<sup>2</sup> acts as a sensitizing agent in the peroxidase reaction and offers the following test which is based upon this fact: To 2–5 c.c. of *raw milk* in a test-tube add 0.1–0.3 c.c. of M/10 hydrogen peroxide and 1 c.c. of a 1 per cent solution of "trikresol." A slight though unmistakable *yellow color* will be observed to develop throughout the solution.

Repeat the test using milk which has been boiled or heated to 80° C. for 10–20 minutes, and cooled, and note that no yellow color is produced.

The color reaction in the case of the raw milk probably results from the oxidation of the cresols by the hydrogen peroxide. The first product of this oxidation<sup>3</sup> then oxidizes the leuco compound, when such is present, and causes the color observed.

(b) *Wilkinson and Peters' Test.*<sup>4</sup>—To 10 c.c. of the milk to be tested add 2 c.c. of a 4 per cent alcoholic solution of benzidine, sufficient acetic acid to coagulate the milk (usually 2–3 drops) and finally 2 c.c. of a 3 per cent solution of hydrogen peroxide. Raw milk yields an immediate blue color. In adding the peroxide it is best to permit it to flow slowly down the wall of the vessel containing the mixture instead of allowing it to mix with the milk. Milk which has been heated to 78° C. or above remains unchanged.

<sup>1</sup> Buckmaster advises the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiaac resin. Guaiaconic acid is a constituent of guaiaac resin.

<sup>2</sup> "Trikresol" is the trade name of an antiseptic which contains the three cresols in approximately equal proportions.

<sup>3</sup> Probably some organic peroxide or quinoid compound.

<sup>4</sup> Wilkinson and Peters: *Z. Nahr-Genussm.*, 16, No. 3, p. 172.



11. **Saturation with Magnesium Sulphate.**—Place about 5 c.c. of milk in a test-tube and saturate with solid magnesium sulphate. What is this precipitate?

12. **Influence of Gastric Rennin on Milk.**—Prepare a series of five tubes as follows:

(a) 5 c.c. of fresh milk + 0.2 per cent HCl (add drop by drop until a precipitate forms).

(b) 5 c.c. of fresh milk + 5 drops of *rennin* solution.

(c) 5 c.c. of fresh milk + 10 drops of 0.5 per cent  $\text{Na}_2\text{CO}_3$ .

(d) 5 c.c. of fresh milk + 10 drops of ammonium oxalate.

(e) 5 c.c. of fresh milk + 5 drops of 0.2 per cent HCl.

Now to each of the tubes (c), (d) and (e) add 5 drops of *rennin* solution. Place the whole series of five tubes at  $40^\circ\text{C}$ . and after 10–15 minutes note what is occurring in the different tubes. Give a reason for each particular result.

13. **Preparation of Caseinogen.**—Fill a large beaker one-third full of *skimmed* (or centrifugated) milk and dilute it with an equal volume of water. Add dilute hydrochloric acid until a flocculent precipitate forms. Stir after each acidification and do not add an excess of the acid as the precipitate would dissolve. Allow the precipitate to settle, decant the supernatant fluid, and reserve it for use in later (14–16) experiments. Filter off the precipitate of caseinogen and remove the excess of moisture by pressing it between filter papers. Transfer the caseinogen to a small beaker, add enough 95 per cent alcohol to cover it and stir for a few moments. Filter, and press the precipitate between filter papers to remove the alcohol. Transfer the caseinogen again to a small *dry* beaker, cover the precipitate with ether and heat on a water-bath for ten minutes, stirring continuously. Filter (reserve the filtrate), and press the precipitate as dry as possible between filter papers. Open the papers and allow the ether to evaporate spontaneously. Grind the precipitate to a powder in a mortar. Upon the caseinogen prepared in this way make the following tests:

(a) *Solubility.*—Try the solubility in the ordinary solvents.

(b) *Millon's Reaction.*—Make the test according to the directions given on page 97.

(c) *Biuret Test.*—Make the test according to directions given on page 98.

(d) *Hopkins-Cole Reaction.*—Make the test according to the directions given on page 98.

(e) *Loosely Combined Sulphur.*—Test for loosely combined sulphur according to the directions given on page 108.

(f) *Fusion Test for Phosphorus*.—Test for phosphorus by fusion according to directions given on page 271.

14. **Coagulable Proteins of Milk**.—Place the filtrate from the original caseinogen precipitate in a casserole and heat, on a wire gauze, over a free flame. As the solution concentrates, a coagulum consisting of *lactalbumin* and *lactoglobulin* will form. Continue to concentrate the solution until the volume is about one-half that of the original solution. Filter off the coagulable proteins (reserve the filtrate) and test them as follows:

(a) *Millon's Reaction*.—Make the test according to the directions given on page 97.

(b) *Biuret Test*.—Make the test according to the directions given on page 98.

(c) *Hopkins-Cole Reaction*.—Make the test according to the directions given on page 98.

15. **Detection of Calcium Phosphate**.—Evaporate the filtrate from the coagulable proteins, on a water-bath, until crystals begin to form.

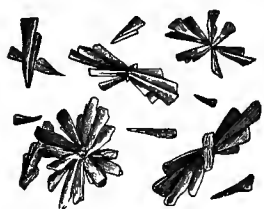


FIG. 81.—CALCIUM PHOSPHATE.

It may be necessary to concentrate to 15 c.c. before any crystallization will be observed. Cool the solution, filter off the crystals (reserve the filtrate), and test them as follows:

(a) *Microscopical Examination*.—Examine the crystals and compare them with those in Fig. 81.

(b) Dissolve the crystals in nitric acid. Test part of the acid solution for phosphates. Render the remainder of the solution slightly alkaline with ammonia, then acidify with acetic acid and add ammonium oxalate. Examine the crystals under the microscope and compare them with those in Fig. 104, p. 363.

16. **Detection of Lactose**.—Concentrate the filtrate from the calcium phosphate until it is of a syrup-like consistency. Allow it to stand over night and observe the formation of crystals of lactose. Make the following experiments.

(a) *Microscopical Examination*.—Examine the crystals and compare them with those in Fig. 80, page 238.

(b) *Fehling's Test*.—Try Fehling's test upon the mother liquor.

(c) *Phenylhydrazine Test*.—Apply the phenylhydrazine test to some of the mother liquor according to the directions given on page 28.

17. **Milk Fat**.—(a) Evaporate the ether filtrate from the caseinogen (Experiment 13) and observe the fatty residue. The milk fat was carried down with the precipitate of caseinogen and was removed when the latter was treated with ether. If centrifugated milk was used in the

preparation of the caseinogen the amount of fat in the ether filtrate may be very small. To secure a larger yield of fat proceed according to directions given under (b) below.

(b). To 25 c.c. of whole milk in an evaporating dish add a little sand or filter paper and evaporate the fluid to dryness on a water-bath. Grind or break up the residue after cooling and extract with ether in a flask. Filter and remove the ether from the filtrate by evaporation. How can you identify fats in the ethereal residue?

18. **Saponification of Butter.**—Dissolve a small amount of butter in alcohol made strongly alkaline with potassium hydroxide. Place the alcoholic-potash solution in a casserole, add about 100 c.c. of water and boil for 10–15 minutes or until the odor of alcohol cannot be detected. Place the casserole in a hood and neutralize the solution with sulphuric acid. Note the odor of volatile fatty acids, particularly butyric acid. Under certain conditions the odor of ethyl butyrate may also be detected.

19. **Detection of Preservatives.**—(a) *Formaldehyde.*

I. *Gallic Acid Test.*—Acidify 30 c.c. of milk with 2 c.c. of normal sulphuric acid and distil. Add 0.2–0.3 c.c. of a saturated alcoholic solution of gallic acid to the *first* 5 c.c. of the distillate, then incline the test-tube and slowly introduce 3–5 c.c. of concentrated sulphuric acid, allowing it to run slowly down the side of the tube. A green ring, which finally changes to blue, is formed at the juncture of the fluids. This is claimed, by Sherman, to be twice as delicate as either the sulphuric acid or the hydrochloric acid test for formaldehyde.

II. *Leach's Hydrochloric Acid Test.*—Mix 10 c.c. of milk and 10 c.c. of concentrated hydrochloric acid containing about 0.002 gram of ferric chloride in a small porcelain evaporating dish or casserole and *gradually* raise the temperature of the mixture, on a water-bath, nearly to the boiling-point, with occasional stirring. If formaldehyde is present a violet color is produced, while a brown color develops in the absence of formaldehyde. In case of doubt the mixture, after having been heated nearly to the boiling-point for about one minute, should be diluted with 50–75 c.c. of water, and the color of the diluted fluid carefully noted, since the violet color if present will quickly disappear. Formaldehyde may be detected by this test when present in the proportion 1:250,000.

(b) *Salicylic and Salicylates.*—Remont's Method.<sup>1</sup> Acidify 20 c.c. of milk with sulphuric acid, shake well to break up the curd, add 25 c.c. of ether, mix thoroughly, and allow the mixture to stand. By means of a pipette remove 5 c.c. of the ethereal extract, evaporate it to dryness, boil the residue with 10 c.c. of 40 per cent alcohol, and cool the alcoholic solution. Make the volume 10 c.c., filter through a *dry* paper if necessary

<sup>1</sup> Sherman's Organic Analysis, First Edition, p. 232.

to remove fat, and to 5 c.c. of the filtrate, which represents 2 c.c. of milk, add 2 c.c. of a 2 per cent solution of ferric chloride. The production of a *purple* or *violet* color indicates the presence of salicylic acid.

This test may form the basis of a quantitative method by diluting the final solution to 50 c.c. and comparing this with standard solutions of salicylic acid. The colorimetric comparisons may be made in a Duboscq colorimeter.

(c) *Hydrogen Peroxide*.—Add 2–3 drops of a 2 per cent aqueous solution of para-phenylenediamine hydrochloride to 10–15 c.c. of milk. If hydrogen peroxide is present a *blue* color will be produced immediately upon shaking the mixture or after allowing it to stand for a few minutes. It is claimed that hydrogen peroxide may be detected by this test when present in the proportion 1:40,000.

(d) *Boric Acid and Borates*.—To the ash, obtained according to the directions given in Experiment 4, page 438, add 2 drops of dilute hydrochloric acid and 1 c.c. of water. Place a strip of turmeric paper in the dish and after allowing it to soak for about one minute remove it and allow it to dry in the air. The presence of boric acid is indicated by the production of a deep *red* color which changes to *green* or *blue* upon treatment with a dilute alkali. This test is supposed to show boric acid when present in the proportion 1:8000.

## CHAPTER XIV.

### EPITHELIAL AND CONNECTIVE TISSUES.

#### EPITHELIAL TISSUE (KERATIN).

The albuminoid *keratin* constitutes the major portion of hair, horn, hoof, feathers, nails, and the epidermal layer of the skin. There is a group of keratins the members of which possess very similar properties. The keratins as a group are insoluble in the usual protein solvents and are not acted upon by the gastric or pancreatic juices. They all respond to the xanthoproteic and Millon reactions and are characterized by containing large amounts of sulphur. Keratin from any of its sources may be prepared in a pure form by treatment, in sequence, with artificial gastric juice, artificial pancreatic juice, boiling alcohol, and boiling ether, from twenty-four to forty-eight hours being devoted to each process.

The percentage composition of some typical keratins is given in the following table:

Source.	Percentage Composition.					
	S	N	C	H	O	
Nails <sup>1</sup> .....	2.80	17.51	51.00	6.94	21.75	
Horn <sup>2</sup> .....	3.20	.....	50.86	6.94	.....	
Human Hair. <sup>3</sup>	Indian.....	4.82	15.40	44.06	6.53	29.19
	Japanese....	4.96	14.64	42.99	5.91	31.50
	Negro.....	4.84	14.90	43.85	6.37	30.04
	Caucasian (adults).	5.22	15.79	44.49	6.44	28.66
	Caucasian (children).	4.93	14.58	43.23	6.46	30.80

The composition of human hair is influenced by its *color* and by the *race, sex, age* and *purity of breeding* of the individual.<sup>3</sup>

<sup>1</sup> Mulder: *Versuch einer allgem. physiol. Chem., Braunschweig, 1844-51.*

<sup>2</sup> Horbaczewski: *Ladenburg's Handwörterbuch d. Chem., 3.*

<sup>3</sup> Rutherford and Hawk: *Jour. Biol. Chem., 3, 459, 1907.*

## EXPERIMENTS ON EPITHELIAL TISSUE.

## Keratin.

Horn shavings or nail parings may be used in the experiments which follow:

1. *Solubility*.—Test the solubility of keratin in the ordinary solvents (see page 27).
2. *Millon's Reaction*.
3. *Xanthoproteic Reaction*.
4. *Adamkiewicz's Reaction*.
5. *Hopkins-Cole Reaction*.
6. *Test for Loosely Combined Sulphur*.

## CONNECTIVE TISSUE.

## I. WHITE FIBROUS TISSUE.

The principal solid constituent of white fibrous connective tissue is the albuminoid collagen. This body is also found in smaller percentage in cartilage, bone, and ligament, but the collagen from the various sources is not identical in composition. In common with the keratins, collagen is insoluble in the usual protein solvents. It differs from keratin in containing less sulphur. One of the chief characteristics of collagen is, according to Hofmeister, the property of being hydrolyzed by boiling acid or water with the formation of *gelatin*. Emmett and Gies<sup>1</sup> claim that under these conditions there is an intramolecular rearrangement of collagen and the resultant gelatin is consequently not the product of hydrolysis. The liberation of ammonia from the collagen during the process apparently confirms this view. Collagen gives Millon's reaction as well as the xanthoproteic and biuret tests.

The form of white fibrous tissue most satisfactory for general experiments is the *tendo Achillis* of the ox. According to Buerger and Gies,<sup>2</sup> the fresh tissue has the following composition:

Water.....	62.87%
Solids.....	37.13
Inorganic matter.....	0.47
Organic matter.....	36.66
Fatty substance (ether-soluble).....	1.04
Coagulable protein.....	0.22
Mucoïd.....	1.28
Elastin.....	1.63
Collagen.....	31.59
Extractives, etc.....	0.90

<sup>1</sup> Emmett and Geis: *Jour. Biol. chem.*, 3, xxxiii (Proceedings), 1907.

<sup>2</sup> Buerger and Gies: *Am. Jour. Physiol.*, 6, 219, 1901.

The mucoid mentioned above is called *tendomucoid*<sup>1</sup> and is a glyco protein. It possesses properties similar to those of other connective-tissue mucoids, *e. g.*, osseomucoid and chondromucoid.

Gelatin, the body which results from the hydrolysis of collagen (see statement of Emmett and Gies above), is also an albuminoid. It responds to nearly all the protein tests. It differs from the keratins and collagen in being easily digested and absorbed. Gelatin is not a satisfactory substitute for the protein constituents of a normal diet, however, since a certain portion of its nitrogen is not available for the uses of the organism. Gelatin from cartilage differs from gelatin from other sources in containing a lower percentage of nitrogen. Tyrosine and tryptophane are not numbered among the decomposition products of gelatin, hence it does not respond to Millon's reaction or the Hopkins-Cole reaction.

#### EXPERIMENTS ON WHITE FIBROUS TISSUE.

The *tendo Achillis* of the ox may be taken as a satisfactory type of the white fibrous connective tissue.

1. **Preparation of Tendomucoid.**—Dissect away the fascia from about the tendon and cut the clean tendon into small pieces. Wash the pieces in running water, subjecting them to pressure in order to remove as much as possible of the soluble protein and inorganic salts. This washing is very important. Transfer the washed pieces of tendon to a flask and add 300 c.c. of *half-saturated* lime water.<sup>2</sup> Shake the flask at intervals for twenty-four hours. Filter off the pieces of tendon and precipitate the mucoid with dilute hydrochloric acid. Allow the mucoid precipitate to settle, decant the supernatant fluid and filter the remainder. Test the mucoid as follows:

(a) *Solubility.*—Try the solubility in the ordinary solvents (see page 27).

(b) *Biuret Test.*—First dissolve the mucoid in potassium hydroxide solution and then add a dilute solution of copper sulphate.

(c) *Test for Loosely Combined Sulphur.*

(d) *Hydrolysis of Tendomucoid.*—Place the remainder of the mucoid in a small beaker, add about 30 c.c. of water and 2 c.c. of dilute hydrochloric acid and boil until the solution becomes dark brown. Cool the solution, neutralize it with concentrated potassium hydroxide, and test by Fehling's test. With a reduction of Fehling's solution and a positive biuret test what do you conclude regarding the nature of tendomucoid?

2. **Collagen.**—This substance is present in the tendon to the extent of about 32 per cent. Therefore in making the following tests upon the

<sup>1</sup> Cutter and Gies: *Am. Jour. Physiol.*, 6, 155, 1901.

<sup>2</sup> Made by mixing equal volumes of *saturated* lime water and water from the faucet.

pieces of tendon from which the mucoid, soluble protein, and inorganic salts were removed in the last experiment, we may consider the tests as being made upon *collagen*.

(a) *Solubility*.—Cut the collagen into very fine pieces and try its solubility in the ordinary solvents (see page 27).

(b) *Millon's Reaction*.

(c) *Biuret Test*.

(d) *Xanthoproteic Reaction*.

(e) *Hopkins-Cole Reaction*.

(f) *Test for Loosely Combined Sulphur*.—Take a *large* piece of collagen in a test-tube and add about 5 c.c. of potassium hydroxide solution. Heat until the collagen is partly decomposed, then add 1-2 drops of lead acetate and again heat to boiling.

(g) *Formation of Gelatin from Collagen*.—Transfer the remainder of the pieces of collagen to a casserole, fill the vessel about two-thirds full of water and boil for several hours, adding water at intervals as needed. By this means the collagen is transformed and a body known as *gelatin* is produced (see p. 247).

3. **Gelatin**.—On the gelatin formed from the transformation of collagen in the above experiment (g), or on gelatin furnished by the instructor make the following tests:

(a) *Solubility*.—Try the solubility in the ordinary solvents (see page 27) and in *hot* water.

(b) *Millon's Reaction*.

(c) *Hopkins-Cole Reaction*.—Conduct this test according to the modification given on page 98.

(d) *Test for Loosely Combined Sulphur*.

Make the following tests upon a *solution* of gelatin in hot water:

(a) *Precipitation by Mineral Acids*.—Is it precipitated by strong mineral acids such as concentrated hydrochloric acid?

(b) *Salting-out Experiment*.—Saturate a little of the solution with solid ammonium sulphate. Is the gelatin precipitated? Repeat the experiment with sodium chloride. What is the result?

(c) *Precipitation by Metallic Salts*.—Is it precipitated by metallic salts such as copper sulphate, mercuric chloride, and lead acetate?

(d) *Coagulation Test*.—Does it coagulate upon boiling?

(e) *Precipitation by Alkaloidal Reagents*.—Is it precipitated by such reagents as picric acid, tannic acid, and trichloroacetic acid?

(f) *Biuret Test*.—Does it respond to the biuret test?

(g) *Bardach's Reaction*.—Does it yield the typical crystals of this reaction? (See page 101.)



(h) *Precipitation by Alcohol*.—Fill a test-tube one-half full of 95 per cent alcohol and pour in a small amount of *concentrated* gelatin solution. Do you get a precipitate? How would you prepare pure gelatin from the *tendo Achillis* of the ox?

II. YELLOW ELASTIC TISSUE (ELASTIN).

The *ligamentum nuchæ* of the ox may be taken as a satisfactory type of the yellow elastic connective tissue. The principal solid constituent of this tissue is *elastin*, a member of the albuminoid group. In common with the keratins and collagen, elastin is an insoluble body and gives the protein color reactions. It differs from keratin principally in the fact that it may be digested by enzymes and that it contains a very small amount of sulphur.

It has recently been demonstrated that elastin has the property of adsorbing pepsin from the gastric juice and thus protecting it so the enzyme can function later in the intestine<sup>1</sup> (see chapter on Gastric Digestion).

Yellow elastic tissue also contains mucoid and collagen but these are present in much smaller amount than in white fibrous tissue, as may be seen from the following percentage composition of the fresh *ligamentum nuchæ* of the ox as determined by Vandegrift and Gies.<sup>2</sup>

Water.....	57.57%
Solids.....	42.43
Inorganic matter.....	0.47
Organic matter.....	41.96
Fatty substance (ether-soluble).....	1.12
Coagulable protein.....	0.62
Mucoid.....	0.53
Elastin.....	31.67
Collagen.....	7.23
Extractives, etc.....	0.80

EXPERIMENTS ON ELASTIN.

I. *Preparation of Elastin (Richards and Gies)*.<sup>3</sup>—Cut the ligament into fine strips, run it through a meat chopper and wash the finely divided material in cold, running water for 24-48 hours. Add an excess of *half-saturated* lime water (see note at the bottom of p. 247) and allow the hashed ligament to extract for 48-72 hours. Decant the lime water, remove all traces of alkali by washing in water and then boil in water with repeated renewals until only traces of protein material can be detected in the wash water. Decant the fluid and boil the ligament in 10 per cent acetic acid for a few hours. Treat the pieces with 5 per cent

<sup>1</sup> Abderhalden and Meyer: *Zeit. physiol. Chem.*, 74, 67, 1911.

<sup>2</sup> Vandegrift and Gies: *Am. Jour. Physiol.*, 5, 287, 1901.

<sup>3</sup> Richards and Gies: *Am. Jour. Physiol.*, 7, 93, 1902.

hydrochloric acid at room temperature for a similar period, extract again in *hot* acetic acid and in *cold* hydrochloric acid. Wash out traces of acid by means of water and then thoroughly dehydrolyze by boiling alcohol and boiling ether in turn. Dry in an air-bath and grind to a powder in a mortar.

2. **Solubility.**—Try the solubility of the finely divided elastin, prepared by yourself or furnished by the instructor, in the ordinary solvents (see page 27). How does its solubility compare with that of collagen?

3. **Millon's Reaction.**

4. **Xanthoproteic Reaction.**

5. **Biuret Test.**

6. **Hopkins-Cole Reaction.**—Conduct this test according to the modification given on page 98.

7. **Test for Loosely Combined Sulphur.**

### III. CARTILAGE.

The principal solid constituents of the matrix of cartilaginous tissue are *chondromucoid*, *chondroitin-sulphuric acid*, *chondroalbumoid* and *collagen*. Chondromucoid differs from the mucoids isolated from other connective tissues in the large amount of chondroitin-sulphuric acid obtained upon decomposition. Besides being an important constituent of all forms of cartilage, chondroitin-sulphuric acid has been found in bone, ligament, the mucosa of the pig's stomach, the kidney of the ox, the inner coats of large arteries and in human urine. It may be decomposed through the action of acid and yields a nitrogenous body known as *chondroitin* and later this body yields *chondrosin*. Chondrosin is also a nitrogenous body and has the power of reducing Fehling's solution more strongly than dextrose. Sulphuric acid is a by-product in the formation of chondroitin, and acetic acid is a by-product in the formation of chondrosin.

Chondroalbumoid is similar in some respects to elastin and keratin. It differs from keratin in being soluble in gastric juice and in containing considerably less sulphur than any member of the keratin group. It gives the usual protein color reactions.

#### EXPERIMENTS ON CARTILAGE.

1. **Preparation of the Cartilage.**—Boil the trachea of an ox in water until the cartilage rings may be completely freed from the surrounding tissue. Use the cartilage so obtained in the following experiments:

2. **Solubility.**—Cut one of the rings into very small pieces and try the solubility of the cartilage in the ordinary solvents (see page 27).

3. **Millon's Reaction.**4. **Xanthoproteic Reaction.**

5. **Hopkins-Cole Reaction.**—Conduct this test according to the modification given on page 98.

6. **Test for Loosely Combined Sulphur.**

7. **Preparation of Cartilage Gelatin.**—Cut the remaining cartilage rings into small pieces, place them in a casserole with water and boil for several hours. Filter while the solution is still hot. Observe that the filtrate soon becomes more or less solid. What is the reason for this? Bring a portion of the material into solution by heat and try the following tests:

(a) *Biuret Test.*

(b) *Bardach's Reaction.*

(c) *Test for Loosely Combined Sulphur.*

(d) To about 5 c.c. of the solution in a test-tube add a few drops of barium chloride. Do you get a precipitate, and if so to what is the precipitate due?

(e) To about 5 c.c. of the solution in a test-tube add a few drops of dilute hydrochloric acid and boil for a few moments. Now add a little barium chloride to this solution. Is the precipitate any larger than that obtained in the preceding experiment? Why?

(f) To the remainder of the solution add a little dilute hydrochloric acid and boil for a few moments. Cool the solution, neutralize with *solid* potassium hydroxide, and try Fehling's test. Explain the result.

## IV. OSSEOUS TISSUE.

Of the solids of bone about equal parts are organic and inorganic matter. The organic portion, called *ossein*, may be obtained by removing the inorganic salts through the medium of dilute acid. Ossein is practically the same body which is termed collagen in the other connective tissues, and in common with collagen yields gelatin upon being boiled with dilute mineral acid.

In common with the other connective tissues bone contains a mucoid and an albumoid. Because of their origin these bodies are called *osseomucoid* and *ossealbumoid*. Osseomucoid, when boiled with hydrochloric acid, yields sulphuric acid and a substance capable of reducing Fehling's solution. The composition of osseomucoid is very similar to that of tendomucoid and chondromucoid (see page 113).

The inorganic basis of the dry, fat-free bone is a chemical substance, not a mixture. This fact is indicated by the uniform composition of the bones of fasting animals as well as by the definite relationship existing

between the elements present. Bones of normal and fasting animals of the same species present no profound differences in percentage composition. The percentage composition of the dry, fat-free femurs of two dogs<sup>1</sup> after the animals had fasted for 104 and 14 days respectively was as follows:

Dog. No.	Length of fast.	Ash.	N.	CaO.	MgO.	P <sub>2</sub> O <sub>5</sub> .
1.	104 days.	61.50	4.6	33.3	0.8	12.80
2.	14 days.	61.56	4.1	33.1	0.9	12.90

The marked uniformity in composition notwithstanding the wide variation in the fasting periods is significant. The tensile strength of the femur of the dog has been found to be at least 25,000 pounds to the square inch<sup>1</sup> whereas that of oak is 10,000 and that of cast iron 20,000 pounds to the square inch.

#### EXPERIMENT ON OSSEOUS TISSUE.

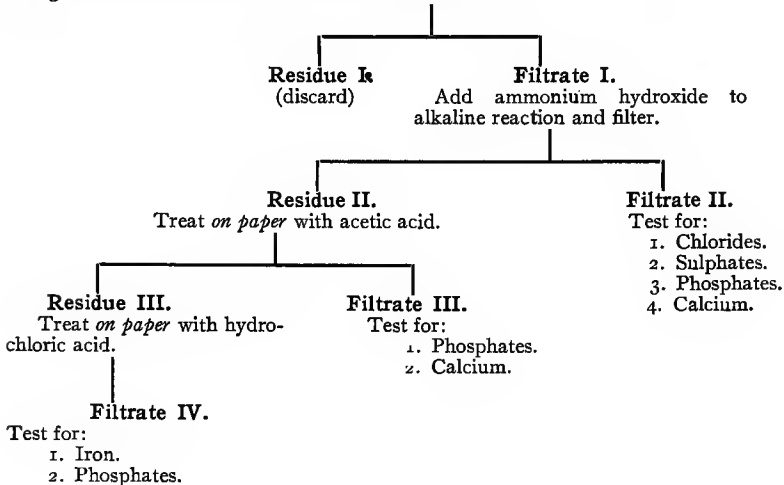
**Qualitative Analysis of Bone Ash.**—Take 1 gram of bone ash in a small beaker and add a little dilute nitric acid. What does the effervescence indicate? Stir thoroughly and when the major portion of the ash is dissolved add an equal volume of water and filter. To the acid filtrate add ammonium hydroxide to alkaline reaction. A heavy white precipitate of phosphates results. (What phosphates are precipitated here by the ammonia?) Filter and test the filtrate for chlorides, sulphates, phosphates, and calcium. Add dilute acetic acid to the precipitate on the paper and test this filtrate for calcium and phosphates. To the precipitate remaining undissolved on the paper add a little dilute hydrochloric acid and test this last filtrate for phosphates and iron.

Reference to the following scheme may facilitate the analysis.

<sup>1</sup> Johnston and Hawk: Unpublished data. For data on a 117-day fast by dog No. 1, see Howe, Mattill and Hawk: *Jour. Biol. Chem.*, 11, 103, 1912.

**BONE ASH.**

Add dilute nitric acid, stir thoroughly and after the major portion of the ash has been brought into solution add a little distilled water and filter.



**V. ADIPOSE TISSUE.**

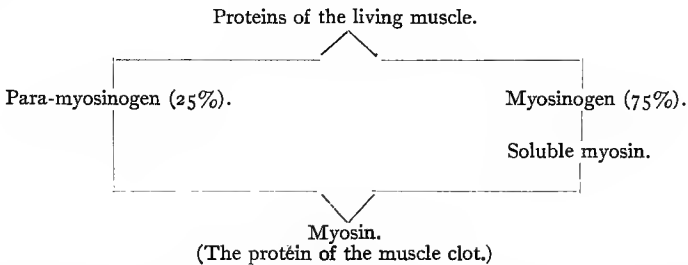
For discussion and experiments see chapter on Fats, page 139.

## CHAPTER XV.

### MUSCULAR TISSUE.

The muscular tissues are divided physiologically into the voluntary (striated) and the involuntary (non-striated or smooth). In the chemical examination of muscular tissue the voluntary form is generally employed. Muscle contains about 25 per cent of solid matter, of which about four-fifths is protein material and the remaining one-fifth extractives and inorganic salts.

The proteins are the most important of the constituents of muscular tissue. In the living muscle we find two proteins, *myosinogen* and *para-myosinogen*. These may be shown to be present in *muscle plasma* expressed from fresh muscles. In common with the plasma of the blood this muscle plasma has the power of coagulating, and the clot formed in this process is called *myosin*. According to Halliburton<sup>1</sup> and others in the onset of *rigor mortis* we have an indication of the formation of this myosin clot within the body. The relation between the proteins of *living* and *dead* muscle is represented graphically by Halliburton as follows:



Of the total protein content of living muscle about 75 per cent is made up by the *myosinogen* and the remaining 25 per cent is para-myosinogen. These proteins may be separated by subjecting the muscle plasma to fractional coagulation in the usual way. Under these conditions the para-myosinogen is found to coagulate at 47° C. and the myosinogen to coagulate at 56° C. It is also claimed by some investigators that it is possible to separate these two proteins by the fractional ammonium sulphate method, but the possibility of making an accurate separation by this method is somewhat doubtful. It is well established that para-myosinogen is a globulin since it responds to certain of the protein precipitation tests and is insoluble in water. Myosinogen, on the contrary,

<sup>1</sup> Halliburton: *Biochemistry of Muscle and Nerve*, 1904, p. 4.

is not a typical globulin since it is soluble in water. It has been called a *pseudo-globulin*. Myosin possesses the globulin characteristics. It is insoluble in water but soluble in the other protein solvents and is precipitated from its solution upon saturation with sodium chloride.

Mellanby has recently reported observations which he claims indicate that there is only *one* protein in muscle and that *rigor mortis* is due to the coagulation of this protein under the combined influences of the salt present in the muscle and the lactic acid developed upon the death of the muscle. He further states that the disappearance of rigor is due to the fact that the lactic acid which is continually formed brings this protein into solution. There is a difference of opinion as to whether true *rigor* ever occurs in connection with non-striated (smooth)<sup>1</sup> muscle.

Our ideas concerning the cause of *rigor* have undergone an important revision quite recently. A very attractive theory has been advanced by Meigs<sup>2</sup> and experimental confirmation has been accorded it by von Fürth and Lenk.<sup>3</sup> According to this theory, *rigor* has no connection with the coagulation of the muscle proteins and may even be hindered or prevented by such coagulation. The cause of *rigor*, from this new view point, lies in the *imbibition of water by the muscle colloids*. It is well known that colloids possess the property of absorbing whatever fluid may be in contact with them. Moreover, the capacity of the colloid for water is increased if the fluid is slightly acid in reaction. Therefore the postmortem production of lactic acid facilitates the imbibition of muscle fluid by the muscle colloids. Under such conditions, the fibers swell, become rigid and the condition known as *rigor mortis* results. The disappearance of rigor is believed to be due to the coagulation of the muscle protein through the agency of the accumulated lactic acid. This change is accompanied by a release of the imbibed water by the colloids, inasmuch as the capacity of a colloid for retaining fluid is lowered by coagulation.

Under the name *extractives* we class a number of muscle constituents which occur in traces in the tissue and may be extracted by water, alcohol, or ether. There are two classes of these extractives, the *non-nitrogenous extractives* and the *nitrogenous extractives*. Grouped under the non-nitrogenous bodies we have *glycogen*, *dextrin*, *sugars*, *lactic acid*, *inosite*,  $C_6H_6(OH)_6$ , and *fat*. In the class of nitrogenous extractives we have *creatine*, *creatinine*, *xanthine*, *hypoxanthine*, *uric acid*, *urea*, *carnine*, *guanine*, *phosphocarnic acid*, *inosinic acid*, *carnosine*, *taurine*, *carnitine*, *novaine*, *ignotine*, *neosine*, *oblitine*, *carnomuscarine* and *methylguanidine* (see formulas on page 260). Not all of these extractives are present in the

<sup>1</sup> Saxl: *Beiträge zur chemischen Physiologie und Pathologie*, 9, 1, 1907.

<sup>2</sup> Meigs: *American Journal of Physiology*, 26, 191, 1910.

<sup>3</sup> von Fürth and Lenk: *Wiener klinische Wochenschrift*, 24, 1079, 1911.

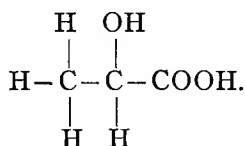
muscles of all species of animals. Other extractives besides those enumerated above have been described and there are undoubtedly still others whose presence remains undetermined. A detailed consideration would, however, be unprofitable in this place.

Glycogen is an important constituent of muscle. The content of this polysaccharide in muscle varies and is markedly decreased by intense muscular activity. It is transformed into sugar and used as fuel. The liver is the organ which stores the reserve supply of glycogen and transforms it into dextrose which is passed into the blood stream and so carried to the working muscle where it is synthesized into glycogen. The glycogen thus formed is then changed into dextrose as the working muscle may need it.

Glycogen is a polysaccharide and has the same percentage composition as starch and dextrin. It resembles starch in forming an opalescent solution and resembles dextrin in being very soluble, in giving a reddish color with iodine and in being dextro-rotatory. Glycogen may be prepared from muscle by extracting with boiling water and then precipitating the glycogen from the aqueous solution by alcohol: dilute or concentrated potassium hydroxide may also be used to extract the glycogen. Glycogen may be prepared in the form of a white, tasteless, amorphous powder. It is completely precipitated from its solution by saturation with solid ammonium sulphate, but is not precipitated by saturation with sodium chloride. It may also be precipitated by alcohol, tannic acid, or ammoniacal basic lead acetate. It has the power of holding cupric hydroxide in solution in alkaline fluids but cannot reduce it. It may be hydrolyzed with the formation of dextrose by dilute mineral acids and is readily digested by amylolytic enzymes.

Mendel and Leavenworth have recently drawn the conclusion, from the examination of embryo pigs, that embryonic structures do not contain exceptionally large amounts of glycogen. The distribution of the glycogen was not observed to differ from that in the adult animal except that the liver of the embryo does not assume its glycogen-storing function early. They further draw the conclusion that the metabolic transformations of glycogen in the embryo and the adult are entirely analogous.

The lactic acid occurring in the muscular tissue of vertebrates is *paralactic* or *sarcolactic acid*,





The reaction of an inactive living muscle is alkaline, but upon the death of the muscle, or after the continued activity of a living muscle, the reaction becomes acid, due to the formation of lactic acid. There is a difference of opinion regarding the origin of this lactic acid. Some investigators claim it to arise from the carbohydrates of the muscle, while others ascribe to it a protein origin.

Among the nitrogenous extratives of muscle, those which are of the most interest in this connection are creatine and the purine bases, xanthine and hypoxanthine. Creatine is found in varying amounts in the muscles of different species, the muscles of birds having shown the largest amount. It has also been found in the blood, the brain, in transudates and in the thyroid gland. Creatine may be crystallized and forms colorless rhombic prisms (Fig. 82, below) which are soluble in warm water and practically

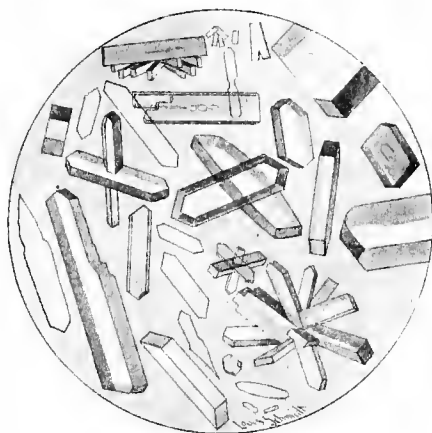


FIG. 82.—CREATINE.

insoluble in alcohol and ether. Upon boiling a solution of creatine with dilute hydrochloric acid it is dehydrolyzed and its anhydride creatinine is formed. The theory that the creatine of ingested meat is transformed into creatinine and excreted in the urine has been proven untenable through the researches of Folin, Klercker, and Wolf and Shaffer. It is now known that under normal conditions the ingestion of creatine in no way influences the excretion of creatinine. In the case of Eck fistula dogs, however, London and Bolyarskii<sup>1</sup> found ingested creatine to increase the output of creatinine in the urine. This finding is of importance as throwing light upon the rôle of the liver in creatine and creatinine metabolism. In this connection it is important to note that there is no *normal* excretion of *endogenous* (see p. 291) creatine, a statement proven by the fact that

<sup>1</sup> London and Bolyarskii: *Zeit. phys. chem.*, 62, 465, 1909.

if no creatine be ingested none will be excreted. Folin<sup>1</sup> has shown that the main bulk of ingested creatine is retained in the body, unless the diet contains a large amount of protein material. Under certain *pathological* conditions the urine may contain *endogenous* creatine which is probably derived from the catabolism of muscular tissue, as Benedict, Mellanby, and Shaffer have suggested.

Amberg and Morrill,<sup>2</sup> Sedgwick,<sup>3</sup> Rose<sup>4</sup> and Folin<sup>5</sup> have shown that creatine is a normal constituent of the urine of infants and children. Folin explains this phenomenon on the basis of the relatively high protein intake, whereas Rose believes it is due to a peculiar carbohydrate metabolism.



FIG. 83.—XANTHINE.

After the drawings of Horbaczewski, as represented in Neubauer and Vogel. (*Ogden*.)

Besides being a normal constituent of muscle, xanthine has been found in the brain, spleen, pancreas, thymus, kidneys, testicles, liver, and in the urine. It may be obtained in crystalline form (Fig. 83, above), but ordinarily it is amorphous. Xanthine is easily soluble in alkalis, less soluble in water and dilute acids, and entirely insoluble in alcohol and ether.

Hypoxanthine occurs ordinarily in those tissues and fluids which contain xanthine. It has been found, unaccompanied by xanthine, in bone marrow and in milk. Unlike xanthine it may be easily crystallized in the form of small, colorless needles. It is readily soluble in alkalis, acids, and boiling water, less soluble in cold water and practically insoluble in alcohol and ether.

The predominating inorganic salt of muscle is potassium phosphate.

<sup>1</sup> Folin: *Hammarsten Festschrift*, p. 15.

<sup>2</sup> Amberg and Morrill: *Jour. Biol. chem.*, 3, 311, 1907.

<sup>3</sup> Sedgwick: *Jour. Am. Med. Ass'n*, 55, 1178, 1910.

<sup>4</sup> Rose: *Jour. Biol. chem.*, 10, 265, 1911.

<sup>5</sup> Folin: *Ibid.*, 11, 253, 1912.

Besides this salt we have present chlorides and salts of sodium, calcium, magnesium, and iron. Sulphates are also present in *traces*.

Mendel and Saiki have made some interesting observations upon the chemical composition of *non-striated* or *smooth* (involuntary) mammalian muscle, such as the urinary bladder and the muscular coat of the stomach of the pig. Hypoxanthine was found to be the predominant purine base present. Creatine and paralactic acid were also isolated. These investigators were unable to demonstrate, definitely, the presence of glycogen in the non-striated muscles studied, but state that "the tissues possess the property of transforming glycogen in the characteristic enzymatic way." The most important part of their investigation consists in a rather complete analysis of the inorganic constituents of these muscles. A notable difference in the relative distribution of the various inorganic constituents was observed, a difference which, according to the authors, "can be accounted for in part only by an admixture of lymph." The comparative composition of the inorganic portion of striated and non-striated muscle and of blood serum for comparison is shown in the appended table:

	Per 100 parts of fresh muscle.							
	K <sub>2</sub> O	Na <sub>2</sub> O	Fe <sub>2</sub> O <sub>3</sub>	CaO	MgO	Cl	P <sub>2</sub> O <sub>5</sub>	H <sub>2</sub> O
Non-striated muscle (Mendel and Saiki).....	0.081	0.328	0.011	0.044	0.007	0.171	0.184	80.6
Skeletal muscle (Katz).....	0.306	0.210	0.008	0.011	0.047	0.048	0.487	72.9
Blood serum (Abderhalden).....	0.027	0.425	.....	0.012	0.004	0.363	0.020	91.8

An interesting comparative study of the ash of the smooth muscle of the stomach of the frog and the striated muscle from the same animal was very recently reported by Meigs and Ryan.<sup>1</sup> Their data indicate "that smooth muscle contains somewhat less potassium and phosphorus and somewhat more sodium and chlorine than the striated muscle of the same animal, but that the differences in these respects between the two tissues are not by any means so marked as has sometimes been supposed." Their average figures for each type of muscle follow:

Muscle.	Per 100 parts of fresh muscle.									
	K	Na	Fe	Ca	Mg	P	Cl	S	Solids	H <sub>2</sub> O
Striated.....	0.350	0.054	0.010	0.028	0.030	0.155	0.066	0.141	20.13	79.87
Smooth.....	0.325	0.073	0.0007	0.004	0.013	0.137	0.120	0.161	17.70	82.30

<sup>1</sup> Meigs and Ryan: *Journal of Biological Chemistry*, II, 401, 1912.

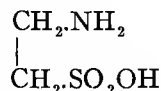
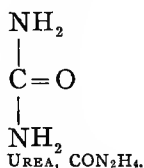
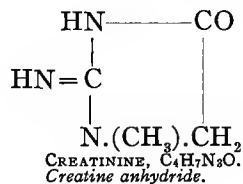
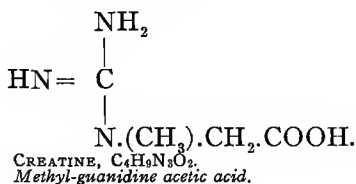
The preparation from which the above data for smooth muscle were obtained were shown by histological examination to consist in large part of smooth muscle fibers.

Muscular tissue is said to contain a reddish pigment called *myohæmatin*, which is a derivative of hæmoglobin.

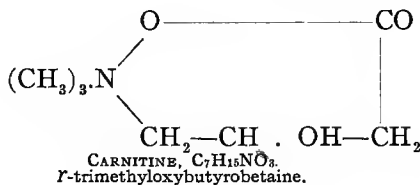
The so-called "fatigue substances" of muscle are carbon dioxide, paralactic acid, and potassium dihydrogen phosphate.

The ordinary commercial "meat extract" is composed principally of the water-soluble constituents of muscle and *contains practically nothing of nutritive value*. The protein material to which meat owes its value as an article of diet is ordinarily practically all removed in the preparation of the extract. Occasionally some preparations are found to contain proteose, which is formed from the meat proteins in the process of preparation.

The structural formulas of some of the nitrogenous extractives of muscle are as follows:



TAURINE,  $\text{C}_2\text{H}_7\text{NSO}_3$ .  
*Amino-ethyl-sulphonic acid.*



Carnosine,  $\text{C}_9\text{H}_{14}\text{N}_4\text{O}_3$ .

Neosine,  $\text{C}_6\text{H}_{17}\text{NO}_2$ .

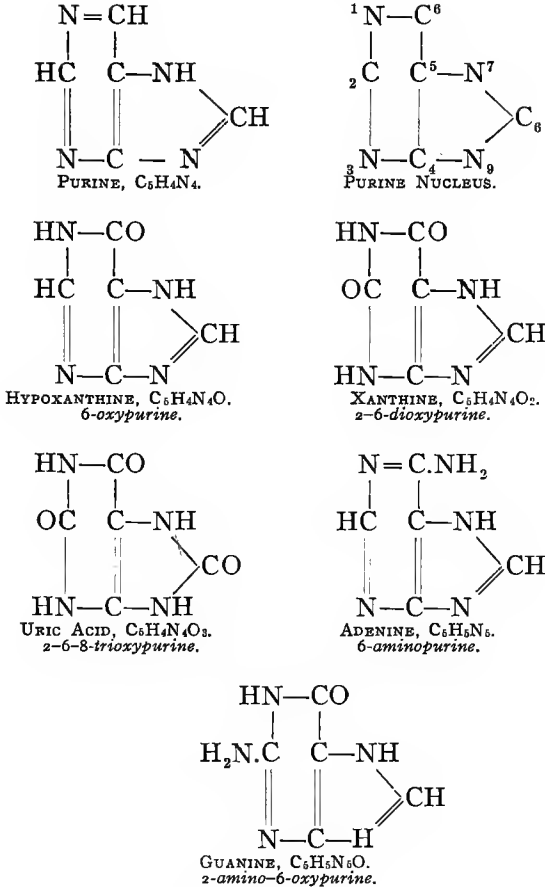
Novaine,  $\text{C}_7\text{H}_{17}\text{NO}_2$ .

Ignotine,  $\text{C}_9\text{H}_{14}\text{N}_4\text{O}_3$ .

Phosphocarnic acid,  $\text{C}_{10}\text{H}_{17}\text{N}_3\text{O}_5$  or  $\text{C}_{10}\text{H}_{15}\text{N}_3\text{O}_5$ .

Inosinic acid,  $(\text{HO})_2 \cdot \text{PO} \cdot \text{O} \cdot \text{CH}_2(\text{CHOH})_3 \cdot \text{CH} : (\text{C}_5\text{H}_3\text{N}_4\text{O})$ .

The following extractives as a group are called *purine bodies*. Their formulas, together with that of *purine* from which they are derived and the hypothetical "purine nucleus" follow:



EXPERIMENTS ON MUSCULAR TISSUE.

I. Experiments on "Living" Muscle.

1. **Preparation of Muscle Plasma (Halliburton).**—Wash out the blood vessels of a freshly killed rabbit with 0.9 per cent sodium chloride. This can best be done by opening the abdomen and inserting a cannula into the aorta. Now remove the skin from the lower limbs, cut away the muscles and divide them into very small pieces by means of a meat chopper. Transfer the pieces of muscle to a mortar and grind them with clean sand and a little 5 per cent magnesium sulphate. Filter off the salted muscle plasma and make the following tests:

(a) *Reaction.*—Test the reaction to litmus phenolphthalein and congo red. What is the reaction of this fresh muscle plasma?

(b) *Fractional Coagulation.*—Place a little muscle plasma in a test-tube and arrange the apparatus for fractional coagulation as explained

on page 106. Raise the temperature very carefully from 30° C. and note any changes which may occur and the exact temperature at which such changes take place. When the first protein (para-myosinogen) coagulates filter it off and then heat the clear filtrate as before, being careful to note the exact temperature at which the next coagulation (myosinogen) occurs. There will probably be a preliminary opalescence in each case before the real coagulation occurs. Therefore do not mistake the real coagulation-point and filter at the wrong time. What are the coagulation temperatures of these two proteins? Which protein was present in greater amount?

(c) *Formation of the Myosin Clot.*—Dilute a portion of the plasma with 3 or 4 times its volume of water and place it on a water-bath or in an incubator at 35° C. for several hours. A typical *myosin clot* should form. Note the muscle serum surrounding the clot. Now test the reaction. Has the reaction changed, and if so to what is the change due? Make a test for lactic acid. What do you conclude?

2. **Preparation of Muscle Plasma (v. Fürth).**—Remove the blood-free muscles of a rabbit as explained above. Finely divide by means of a meat chopper and grind in a mortar with a little clean sand and some 0.9 per cent sodium chloride. Wrap portions of the muscle in muslin and press thoroughly by means of a tincture press or lemon squeezer. Filter and make the tests according to the directions given in the last experiment.

3. **“Fuchsin-frog” Experiment.**—Inject a saturated aqueous solution of Fuchsin “S” into the lymph spaces of a frog two or three times daily for one or two days, in this way thoroughly saturating the tissues with the dye. Pith the animal (insert a heavy wire or blunt needle through the occiput atlantoid membrane), remove the skin from both hind legs and expose the sciatic nerve in one of them. Insert a small wire hook through the jaws of the frog and suspend the animal from an ordinary clamp or iron ring. Pass electrodes under the exposed sciatic nerve, and after tying the other leg to prevent any muscular movement, stimulate the exposed nerve by means of *make* and *break* shocks from an induction coil. The stimulated leg responds by pronounced muscular contractions, whereas the tied leg remains inactive. Continue the stimulation until the muscles are fatigued. The muscular activity has caused the production of *lactic acid* and this in turn has reacted with the injected fuchsin to cause a *pink or red* color to develop. The muscles of the inactive leg still remain unchanged in color.

The normal color of the Fuchsin “S” when injected was red, but upon being absorbed it became colorless through the action of the alkalinity of the blood. Upon stimulating the muscles, however, as above explained,

lactic acid was formed and this acid reacted with the fuchsin and again produced the original color of the dye.

## II. Experiments on "Dead" Muscle.

1. **Preparation of Myosin.**—Take 25 grams of finely divided lean beef which has been carefully washed to remove blood and lymph constituents and place it in a beaker with 10 per cent sodium chloride. Stir occasionally for several hours. Strain off the meat pieces by means of cheese cloth, filter the solution and saturate it with sodium chloride in substance. Filter off the precipitate of *myosin* and make the tests as given below. This filtration will proceed very slowly. Myosin collects as a film on the sides of the filter paper and may be removed and tested before the entire volume of fluid has been filtered. If this precipitate remains for any length of time on the paper in contact with the air it will become transformed into the protean *myosan*. Test the myosin precipitate as follows:

(a) *Solubility.*—Try its solubility in the ordinary solvents. Is myosin an albumin or a globulin?

(b) *Xanthoproteic Reaction.*—See page 97.

(c) *Coagulation Test.*—Suspend a little of the myosin in water in a test-tube and heat to boiling for a few moments. Now remove the suspended material and try its solubility in 10 per cent sodium chloride. What property does this experiment show myosin to possess?

Test the filtrate from the original myosin precipitate as follows:

(a) *Biuret Test.*—What does this show?

(b) Place a little of the solution in a test-tube and heat to boiling. At the boiling-point add a drop of dilute acetic acid and filter. Test this filtrate for proteose with picric acid. Is any proteose present? Saturate another portion of the filtrate with ammonium sulphate and test for peptone in the usual way. (see page 120) Do you find any peptone? From your experiments on "living" and "dead" muscle what are your ideas regarding the proteins of muscle?

2. **Preparation of Glycogen.**—Grind a few oysters or scallops<sup>1</sup> in a mortar with sand. Transfer to an evaporating dish, add water, and boil for 20 minutes. Note the opalescence of the solution. At the boiling-point faintly acidify with acetic acid. Why is this acid added? Filter, and divide the filtrate into two parts. Test one part of the filtrate as follows:

(a) *Iodine Test.*—To 50 c.c. of the solution in a test-tube add 5–10 drops of iodine solution and 2–3 drops of 10 per cent sodium chloride.

<sup>1</sup> Glycogen may also be prepared from the liver of an animal which has been fed a high carbohydrate diet for 1–2 days previously. The best yield of glycogen can, however, generally be obtained from scallops.

What do you observe? Is this similar to the iodine test upon any other body with which we have had to deal?

If difficulty is experienced in securing a satisfactory iodine test proceed as follows: Make equal volumes of glycogen solution acid in reaction with hydrochloric acid. Boil one solution to hydrolyze the glycogen. Add equal volumes of iodine solution to each and note the more pronounced iodine reaction in the unhydrolyzed solution.

(b) *Reduction Test*.—Does the solution reduce Fehling's solution?

(c) *Hydrolysis of Glycogen*.—Add 10 drops of concentrated hydrochloric acid to 10 c.c. of the solution and boil for 10 minutes. Cool the solution, neutralize with *solid* potassium hydroxide and test with Fehling's solution. Does it still fail to reduce Fehling's solution? If you find a reduction how can you prove the identity of the reducing substance?

(d) *Influence of Saliva*.—Place 5 c.c. of the solution in a test-tube, add 5 drops of saliva and place on the water-bath at 45° C. for 10 minutes. Does this now reduce Fehling's solution?

To the second part of the glycogen filtrate add 3–4 volumes of 95 per cent alcohol. Allow the glycogen precipitate to settle, decant the supernatant fluid, and filter the remainder. Heat the glycogen on a water-bath to remove the alcohol, then subject it to the following tests:

(a) *Solubility*.—Try its solubility in the ordinary solvents.

(b) *Iodine Test*.—Place a small amount of the glycogen in a depression of a test-tablet and add 2–3 drops of dilute iodine solution and a trace of a sodium chloride solution. The same wine-red color is observed as in the iodine test upon the glycogen solution.

### Separation of Extractives from Muscle.

1. **Creatine**.—Dissolve about 10 grams of a commercial extract of meat in 200 c.c. of warm water. Precipitate the inorganic constituents by neutral lead acetate, being careful not to add an excess of the reagent. Write the equations for the reactions taking place here. Allow the precipitate to settle, then filter and remove the excess of lead in the *warm* filtrate by hydrogen sulphide. Filter while the solution is yet warm, evaporate the clear filtrate to a syrup, and allow it to stand at least 48 hours in a cool place. Crystals of creatine should form at this point. Examine under the microscope (Fig. 82, page 257). Treat the syrup with 200 c.c. of 88 per cent alcohol, stir well with a glass rod to bring all soluble material into solution, and then filter. The purine bases have been dissolved and are in the filtrate, whereas the creatine crystals were insoluble in the 88 per cent alcohol and remain on the filter paper. Wash the crystals with 88 per cent alcohol, then remove them and bring



them into solution in a little hot water. Decolorize the solution by animal charcoal and concentrate it to a small volume. Allow the solution to cool and note the separation of colorless crystals of creatine. Examine these crystals under the microscope and compare them with those reproduced in Fig. 82, page 257.

2. **Hypoxanthine.**—Evaporate the alcoholic filtrate from the creatine to remove the alcohol. Make the solution ammoniacal and add ammoniacal silver nitrate until precipitation ceases. The precipitate consists principally of *hypoxanthine silver* and *xanthine silver*. Collect these silver salts on a filter paper and wash them with water. Place the precipitate and paper in an evaporating dish and boil for one minute with nitric acid having a specific gravity of 1.1. Filter while *hot* through a

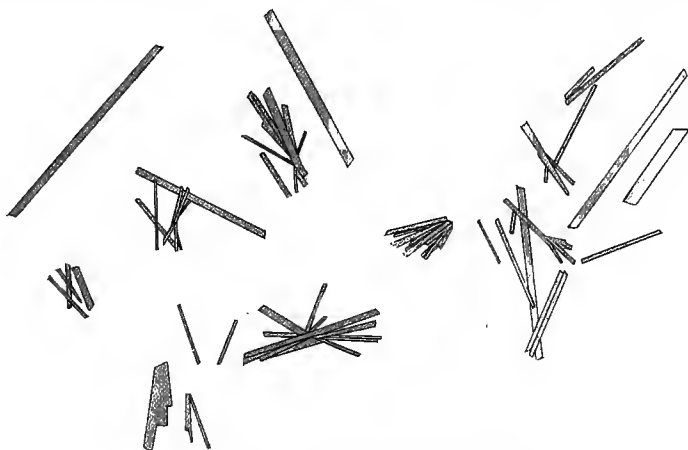


FIG. 84.—HYPOXANTHINE SILVER NITRATE.  
(Drawn from a student preparation by Mr. E. F. Hirsch).

double paper, wash with the same strength of nitric acid and allow the solution to cool. By this treatment with nitric acid *hypoxanthine silver nitrate* and *xanthine silver nitrate* have been formed. The former is insoluble in the cold solution and separates on standing. After standing several hours filter off the hypoxanthine silver nitrate and wash with water until the wash-water is only slightly acid in reaction. Examine the crystals of *hypoxanthine silver nitrate* under the microscope and compare them with those in Fig. 84, above. Now wash the crystals from the paper into a beaker with a little water and warm the liquid. Remove the silver by hydrogen sulphide and filter. By this means *hypoxanthine nitrate* has been formed and is present in the filtrate. Concentrate on a water-bath to drive off hydrogen sulphide and render the solution slightly alkaline with ammonia. Warm for a time, to remove

the free ammonia, filter, concentrate the filtrate to a small volume and allow it to stand in a cool place. Hypoxanthine should crystallize in small colorless needles. Examine the crystals under the microscope.

3. **Xanthine.**—To the filtrate from the above experiment containing the *xanthine silver nitrate* add ammonia in excess. (The crystalline form of xanthine silver nitrate is shown in Fig. 85, below.) A brownish-red precipitate of *xanthine silver* forms. Treat this suspended precipitate with hydrogen sulphide (do not use an excess of hydrogen sulphide), warm the mixture for a few moments and filter while hot. Concentrate

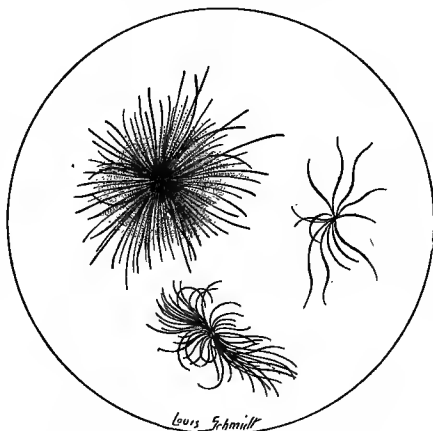


FIG. 85.—XANTHINE SILVER NITRATE.

the filtrate to a small volume and put away in a cool place for crystallization (Fig. 83, p. 258). To obtain xanthine in crystalline form special precautions are generally necessary. Evaporate the solution to dryness. Make the following tests on the crystals or residue:

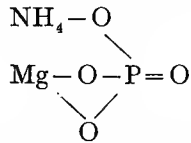
(a) *Xanthine Test.*—Place about one-half of the crystalline or amorphous material in a small evaporating dish, add a few drops of concentrated nitric acid and evaporate to dryness very carefully on a water-bath. The yellow residue upon moistening with caustic potash becomes red in color and upon further heating assumes a purplish-red hue. Now add a few drops of water and warm. In this way a yellow solution results which yields a red residue upon evaporation. How does this differ from the Murexide test upon uric acid?

(b) *Weidel's Reaction.*—By gently heating bring the remainder of the xanthine crystals or residue into solution in bromine-water. Evaporate the solution to dryness on a water-bath. Remove the stopper from an ammonia bottle and by blowing across the mouth of the bottle direct the fumes of ammonia so that they come in contact with the dry residue. Under these conditions the presence of xanthine is shown by the residue

assuming a red color. A somewhat brighter color may be obtained by using a trace of nitric acid with the bromine-water. By the use of this modification, however, we may get a positive reaction with bodies other than xanthine.

#### HÜRTHLE'S EXPERIMENT.

Tease a very small piece of frog's muscle on a microscopical slide. Expose the slide to ammonia vapor for a few moments, then adjust a cover glass, and examine the muscle fibers under the microscope. Note the large number of crystals of ammonium magnesium phosphate,



distributed everywhere throughout the muscle fiber, thus demonstrating the abundance of phosphates and magnesium in the muscle (Fig. 101, page 319.)

## CHAPTER XVI.

### NERVOUS TISSUE.

In common with the other solid tissues of the body, nervous tissue contains a large amount of water. The percentage of water present depends upon the particular form of nervous tissue but in all forms it is invariably greater in the gray matter than in the white. Embryonic nervous tissues also contain a larger percentage of water than the tissues of adult life. The gray matter of the brain of the foetus, for instance, contains about 92 per cent of water, whereas the gray matter of the brain of the adult contains but 83-84 per cent of the fluid.

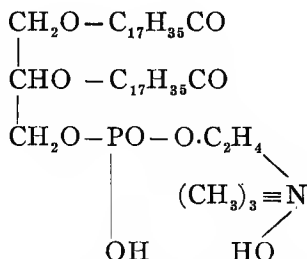
Among the solid constituents of nervous tissue are *proteins*, *cholesterol*, *cerebrosides* (cerebrin, etc.), *lecithin*, *kephalin*, *protagon* (?), *para-nucleoprotagon*, *nuclein*, *neurokeratin*, *collagen*, *extractives*, and *inorganic salts*. The proteins are present in the greatest amount and comprise about 50 per cent of the total solids. Three distinct proteins, two globulins, and a nucleoprotein, have been isolated from nervous tissue. The globulins coagulate at 47° C. and 70-75° C., respectively, while the nucleoprotein coagulates at 56-60° C. This nucleoprotein contains about 0.5 per cent of phosphorus (Halliburton, Levene). Nervous tissue is composed of a relatively large quantity of a variety of compounds which collectively may be grouped under the term "lipoid"—substances resembling the fats in some of their physical properties and reactions but distinct in their composition. We will class cholesterol, the cerebrosides and the phosphorized fats as lipoids.

The consideration of lipoids (or *lipins*<sup>1</sup>) is assuming added importance. These substances constitute one of the two great groups of *tissue colloids*, the proteins being the remaining group. So far as structure and chemical properties are concerned the various classes of lipoids are entirely unlike.

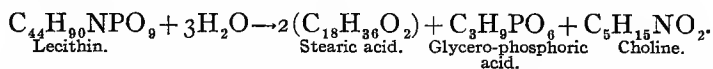
The group of *phosphorized fats* are very important constituents of nervous tissue. The best known members of this group are *lecithin*, *protagon* (?) and *kephalin*. Lecithin occurs in larger amount than the other members of the group, has been more thoroughly studied than the others and is apparently of greater importance. Upon decomposition lecithin yields *fatty acid*, *glycero-phosphoric acid*, and *choline*.

<sup>1</sup> Rosenbloom and Gies: *Biochemical Bulletin*, 1, 51, 1911. The term lipoid was introduced by Overton (Studien über die Narkose, Jena, 1901, Gustav Fischer.)

Each lecithin molecule contains two fatty acid radicals which may be those of the same or different fatty acids. Thus we have different lecithins depending upon the particular fatty acid radicals which are present in the molecule. The formula of a typical lecithin would be the following:

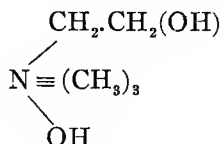


This lecithin would be called distearyl-lecithin or *choline-distearyl-glycero-phosphoric acid*. Upon decomposition the molecule splits according to the following reaction:



The lecithins are not confined to the nervous tissues but are found in nearly all animal and vegetable tissues. Lecithin is a primary constituent of the cell. It is soluble in chloroform, ether, alcohol, benzene, and carbon disulphide. The chloroform or alcohol-ether solution may be precipitated by acetone. Lecithin may be caused to crystallize in the form of small plates by cooling the alcoholic solution to a low temperature. It has the power of combining with acids and bases, and the hydrochloric acid combination has the power of forming a double salt with platinic chloride.

Choline, as was indicated above, is one of the decomposition products of lecithin. It is *trimethyl-oxylethyl-ammonium hydroxide* and has the following formula:



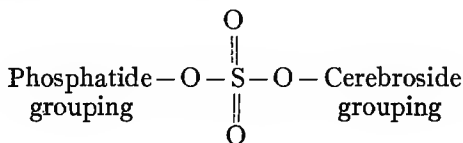
Recent researches have shown that great importance is to be attached to the detection of choline in the cerebro-spinal fluid and the blood in certain cases of degenerative disease of the nervous system. In this connection tests for choline (see p. 273) are of interest and value.

Protagon, another nitrogenous phosphorized substance is a body over which there has been much discussion. Upon decomposition it

is said by some investigators to yield cerebrin and the decomposition products of lecithin. It has been shown by Posner and Gies<sup>1</sup> as well as by Rosenheim and Tebb<sup>2</sup> that protagon is a mixture and has no existence as a chemical individual. Koch<sup>3</sup> very recently reported data obtained from purified preparations which indicate that protagon contains at least three substances: "a phosphatide containing cholin, a cerebroside containing sugar, a complex combination of a cholin-free phosphatide with a cerebroside to which an ethereal sulphuric acid group is attached." On the basis of his data, he believed the term protagon to have no chemical significance. He proposed the term sulphatide. Koch's preparation analyzed as follows (per cent):

Choline	Sugar	Nitrogen	Phosphorus	Sulphur
1.0	12.0	2.3	1.7	1.9

He suggested the following structure:



Kephalin is the third member of the group of phosphorized fats. It is precipitated from its acetone-ether extract by alcohol. It contains about 4 per cent of phosphorus and has been given the formula  $\text{C}_{42}\text{H}_{79}\text{-NPO}_{13}$ . Kephalin may be a stage in lecithin metabolism.

The cerebrosides are substances containing nitrogen but no phosphorus, and are important constituents of the white matter of nervous tissue. Certain ones have also been found in the spleen, pus, and in egg yolk. They may be extracted from the tissue by boiling alcohol and are insoluble in cold alcohol, cold and hot ether, and in water and dilute alkalis. The cerebroside termed cerebrin is a mixture containing phrenosin (pseudo-cerebrin or cerebrin), a body yielding the carbohydrate galactose on decomposition.

Cholesterol, one of the primary cell constituents, is present in fairly large amount in nervous tissue. It is a mon-atomic alcohol containing at least *one* double bond and possesses the formula  $\text{C}_{27}\text{H}_{45}\text{OH}$  or  $\text{C}_{27}\text{-H}_{43}\text{OH}$ . There is still some uncertainty as to the exact structure of cholesterol. It may possess a terpene structure. It was formerly called a "non-saponifiable fat" but since it is not changed in any way by boiling alkalis it is not a fat. It is soluble in ether, chloroform, benzene, and hot alcohol. It crystallizes in the form of thin, colorless, trans-

<sup>1</sup> Posner and Gies: *Journal of Biological Chemistry*, 1, 59, 1905-6.

<sup>2</sup> Rosenheim and Tebb: *Journal of Physiology*, 36 and 37, 1907-8.

<sup>3</sup> Koch: *Journal of Biological Chemistry*, 11, March, 1912, Proceedings.

parent plates (Fig. 43, p. 166). Cholesterol occurs abundantly in one form of biliary calculus. It has also been found in feces, wool fat, egg yolk, and milk, frequently in the form of its esters of higher fatty acids. It is generally believed that the cholesterol present in the animal body has its origin in the vegetable kingdom. However evidence has recently been submitted<sup>1</sup> indicating a synthesis of cholesterol under certain conditions in the animal body.

Paranucleoprotagon is a phosphorized substance originally isolated from brain tissue by Ulpiani and Lelli and recently reinvestigated by Steel and Gies. It is said to possess lecithoprotein characteristics.

Nervous tissue yields about 1 per cent of ash which is made up in great part of alkaline phosphates and chlorides.

#### EXPERIMENTS ON THE LIPOIDS OF NERVOUS TISSUE.<sup>2</sup>

1. **Preparation of Lecithin.**—Treat the macerated brain of a sheep with ether and allow it to stand in the cold for 48–72 hours. The cold ether will extract lecithin and cholesterol. Filter and add acetone to the filtrate to precipitate the lecithin. Filter off the lecithin and test it as follows:

(a) *Microscopical Examination.*—Suspend a small portion in a drop of water on a slide and examine under the microscope.

(b) *Osmic Acid Test.*—Treat a small portion with osmic acid. What happens?

(c) *Acrolein Test.*—Make the acrolein test according to directions on page 143.

(d) *“Fusion” Test for Phosphorus.*—Place some of the lecithin prepared above in a small porcelain crucible, add a suitable amount of a fusion mixture composed of potassium hydroxide and potassium nitrate (5:1) and heat *carefully* until the resulting mixture is colorless. Cool, dissolve the mass in a little warm water, acidify with nitric acid, heat to boiling, and add a few cubic centimeters of molybdic solution. In the presence of phosphorus a yellow precipitate forms. What is it?

2. **Preparation of Cholesterol.**—Place a small amount of macerated brain tissue under ether and stir occasionally for one hour. Filter, evaporate the filtrate to dryness on a water-bath, and test the cholesterol according to directions given below. (If it is desired, the ether extract

<sup>1</sup> Klein: *Biochem. Zeit.*, 30, 465, 1910.

<sup>2</sup> Preparation of So-called Protagon.—Macerate the brain of a sheep, treat with 85 per cent alcohol and warm on a water-bath at 45° C. for two hours. Filter *hot* into a bottle or strong flask and cool to 0° C. for one-half hour by means of a freezing mixture. By this procedure both protagon and cholesterol are caused to precipitate. Filter the cold solution rapidly and treat the precipitate on the paper with ice cold ether to dissolve out the cholesterol. The protagon may now be redissolved in warm 85 per cent alcohol from which solution it will precipitate upon cooling.

from the so-called protagon, or the ether-acetone filtrate from the lecithin may be used for the isolation of cholesterol. In these cases it is simply necessary to evaporate the solution to dryness on a water-bath.) Upon the cholesterol prepared by either of the above methods make the following tests:

(a) *Microscopical Examination*.—Examine the crystals under the microscope and compare them with those in Fig. 43, page 166.

(b) *Iodine-sulphuric Acid Test*.—Place a few crystals of cholesterol in one of the depressions of a test-tablet and treat with a drop of concentrated sulphuric acid and a drop of a very dilute solution of iodine. A play of colors, consisting of violet, blue, green, and red, results.

(c) *The Liebermann-Burchard Test*.—Dissolve a few crystals of cholesterol in 2 c.c. of chloroform in a *dry* test-tube. Now add 10 drops of acetic anhydride and 1–3 drops of concentrated sulphuric acid. The solution becomes red, then blue, and finally bluish-green in color.

(d) *Salkowski's Test*.—Dissolve a few crystals of cholesterol in a little chloroform and add an equal volume of concentrated sulphuric acid. A play of colors from bluish-red to cherry-red and purple is noted in the chloroform, while the acid assumes a marked green fluorescence.

(e) *Schiff's Reaction*.—To a little cholesterol in an evaporating dish add a few drops of Schiff's reagent.<sup>1</sup> Evaporate to dryness over a low flame and observe the reddish-violet residue which changes to a bluish-violet.

(f) *Phosphorus*.—Test for phosphorus according to directions given on page 271. Is phosphorus present?

3. **Preparation of Cerebrin**.—Treat the macerated brain tissue, in a flask, with 95 per cent alcohol and boil on a water-bath for one-half hour, keeping the volume constant by adding fresh alcohol as needed. Filter the solution *hot* and stand the *cloudy* filtrate away for twenty-four hours. (If the filtrate is not cloudy concentrate it upon the water-bath until it is so.) Filter off the cerebrin and test it as follows:

(a) *Microscopical Examination*.—Suspend a small portion in a drop of water on a slide and examine under the microscope.

(b) *Solubility*.—Try the solubility of cerebrin in the usual solvents and in hot and cold alcohol and hot and cold ether.

(c) *Phosphorus*.—Test for phosphorus according to directions on page 271. How does the result compare with that on lecithin?

(d) Place a little cerebrin on platinum foil and warm. Note the odor.

<sup>1</sup> Schiff's reagent consists of a mixture of three volumes of concentrated sulphuric acid and one volume of 10 per cent ferric chloride.



(e) *Hydrolysis of Cerebrin*.—Place the remaining cerebrin in a small evaporating dish, add equal volumes of water and dilute hydrochloric acid, and boil for one hour. Cool, neutralize with *solid* potassium hydroxide, filter, and test with Fehling's solution. Is there any reduction, and if so how do you explain it?

4. **Tests for Choline.** (a) *Rosenheim's Periodide Test*.—Prepare an alcoholic extract of the fluid under examination, and after evaporation, apply Rosenheim's iodo-potassium iodide solution<sup>1</sup> to a little of the residue. In a short time dark brown plates and prisms of *choline periodide* begin to form and may be detected by means of the microscope. Occasionally they are large enough to be visible to the naked eye. They somewhat resemble crystals of hæmin (see p. 211). If the slide be permitted to stand, thus allowing the fluid to evaporate, the crystals will disappear and leave brown oily drops. They will reappear, however, upon the addition of fresh iodine solution. v. Staněk claims that this choline compound has the formula  $C_5H_{14}NOI_3$ .

(b) *Rosenheim's Bismuth Test*.—Extract the fluid under examination with absolute alcohol, evaporate, and re-extract the residue. Repeat the extraction several times. Dissolve the final residue in 2–3 c.c. of water and add a drop of Kraut's reagent.<sup>2</sup> Choline is indicated by the appearance of a bright brick-red precipitate.

<sup>1</sup> Prepared by dissolving 2 grams of iodine and 6 grams of potassium iodide in 100 c.c. water.

<sup>2</sup> Dissolve 272 grams of potassium iodide in water and add 80 grams of bismuth subnitrate dissolved in 200 grams of nitric acid (sp. gr. 1.18). Permit the potassium nitrate to crystallize out, then filter it off and make the filtrate up to 1 liter with water.

## CHAPTER XVII.

### URINE: GENERAL CHARACTERISTICS OF NORMAL AND PATHOLOGICAL URINE.

**Volume.**—The volume of urine excreted by normal individuals during any definite period fluctuates within very wide limits. The average output for twenty-four hours is placed by German writers between 1500 and 2000 c.c. This value is not strictly applicable to conditions in America, however, since it has been found that the average normal excretion of the adult male American falls within the lower values of 1000–1200 c.c. The volume-excretion is influenced greatly by the diet, particularly by the ingestion of fluids.

Certain pathological conditions cause the output of urine for any definite period to depart very decidedly from the normal output. Among the pathological conditions in which the volume of urine is *increased* above normal are the following: Diabetes mellitus, diabetes insipidus, certain diseases of the nervous system, contracted kidney, amyloid degeneration of the kidney, and in convalescence from acute diseases in general. Many drugs such as calomel, digitalis, acetates, and salicylates also increase the volume of the urine excreted. A *decrease* from the normal is observed in the following pathological conditions: Acute nephritis, diseases of the heart and lungs, fevers, diarrhœa, and vomiting.

**Color.**—Normal urine ordinarily possesses a yellow tint, the depth of the color being dependent in part upon the density of the fluid. The color of normal urine is due principally to a pigment called *urochrome*: traces of *hæmatoporphyrin*, *urobilin*, and *uroerythrin* have also been detected. Under pathological conditions the urine is subject to pronounced variations in color and may contain many varieties of pigments. Under such circumstances the urine may vary in color from an extremely light yellow to a very dark brown or black. Vogel has constructed a color chart which is of some value for purposes of comparison. The nature and origin of the chief variations in the urinary color are set forth in tabular form by Halliburton as follows:

Color.	Cause of Coloration.	Pathological Condition.
Nearly colorless.....	Dilution, or diminution of normal pigments.	Nervous conditions: hydruria, diabetes insipidus, granular kidney.
Dark yellow to brown-red....	Increase of normal, or occurrence of pathological, pigments.	Acute febrile diseases.
Milky.....	Fat globules.....	Chyluria.
	Pus corpuscles.....	Purulent diseases of the urinary tract.
Orange.....	Excreted drugs.....	Santonin, chrysophanic acid.
Red or reddish.....	Hæmatoporphyrin.....	Hæmorrhages, or hæmoglobinuria.
	Unchanged hæmoglobin.....	
Brown to brown-black.....	Pigments in food (logwood, madder, bilberries, fuchsin).	
	Hæmatin.....	Small hæmorrhages.
	Methæmoglobin.....	Methæmoglobinuria.
	Melanin.....	Melanotic sarcoma.
Greenish-yellow, greenish-brown, approaching black.	Hydrochinon and catechol....	Carbolic-acid poisoning.
	Bile-pigments.....	Jaundice.
Dirty green <sup>1</sup> or blue.....	A dark-blue scum on surface, with a blue deposit, due to an excess of indigo-forming substances.	Cholera, typhus; seen especially when the urine is putrefying.
Brown-yellow to red-brown, becoming blood-red upon adding alkalis.	Substances contained in senna, rhubarb, and chelidonium which are introduced into the system.	

**Transparency.**—Normal urine is ordinarily perfectly clear and transparent when voided. On standing for a variable time, however, a cloud (nubecula) consisting principally of nucleoprotein or mucoid (see p. 308) and epithelial cells forms. A turbidity due to the precipitation of phosphates is normally noted in urine passed after a hearty meal. The urine obtained 2–3 hours after a meal or later is ordinarily free from turbidity. Permanently turbid urines ordinarily arise from pathological conditions.

**Odor.**—The odor of normal urine is of a faint, aromatic type. The bodies to which this odor is due are not well known, but it is claimed by some investigators to be due, at least in part, to the presence of minute amounts of certain volatile organic acids. When the urine undergoes decom-

<sup>1</sup> This dirty green or blue color also occurs after the use of methylene blue in the organism.

position, *e. g.*, in alkaline fermentation, a very unpleasant ammoniacal odor is evolved. All urines are subject to such decomposition if allowed to stand for a sufficiently long time. Under normal conditions the urine very often possesses a peculiar odor due to the ingestion of some certain drug or vegetable. For instance, cubebs, copaiba, myrtol, saffron, tolu, and turpentine each imparts a somewhat specific odor to the urine. After the ingestion of asparagus, the urine also possesses a typical odor.

**Frequency of Urination.**—The frequency of urination varies greatly in different individuals but in general is dependent upon the amount of fluid in the bladder. In pathological conditions an inflammatory affection of the urinary tract or any disturbance of the innervation of the bladder will influence the frequency. Affections of the spinal cord which lead to an increased irritability of the bladder or a weakening of the sphincter will result in increasing the frequency of urination.

**Reaction.**—The mixed twenty-four hour urinary excretion of a normal individual ordinarily possesses an acid reaction to litmus. This acidity is now believed to be due to the presence of various acidic radicals and not to the presence of *sodium di-hydrogen phosphate* as was formerly held (see Phosphates, p. 317). This conclusion is reinforced by the observation that urine may be divided into two portions, one part consisting almost entirely of inorganic matter, including practically *all of the phosphates* and having an *alkaline reaction*, the other containing practically all of the *organic substances* and no phosphates and having an *acid reaction*. The acidity imparted to the urine by any particular acid depends entirely upon the extent to which the acid is dissociable, since it is the hydrogen ion which is responsible for the acid reaction.

The composition of the food is perhaps the most important factor in determining the reaction of the urine. The reaction ordinarily varies considerably according to the time of day the urine is passed. For instance, for a variable length of time after a meal the urine may be neutral or even alkaline in reaction to litmus, owing to the claim of the gastric juice upon the acidic radicals to further the formation of hydrochloric acid for use in carrying out the digestive secretory function. This change in reaction is known as the *alkaline tide* and is common to perfectly healthy individuals. The urine may also become temporarily alkaline in reaction to litmus, as the result of ingesting alkaline carbonates or certain salts of tartaric and citric acids which may be transformed into carbonates within the organism. Normal urine upon standing for some time becomes alkaline in reaction to litmus, owing to the inception of alkaline or ammoniacal fermentation through the agency of micro-organisms. This fermentation has no especial diagnostic value except in cases where the urine has undergone this change *within the*

*organism* and is voided in the decomposed state. Ammoniacal fermentation is ordinarily due to cystitis or occurs as the result of infection in the process of catheterization. A microscopical examination of such urine (Fig. 86, below) shows the presence of *ammonium magnesium phosphate* crystals, *amorphous phosphates*, and not infrequently *ammonium urate*.

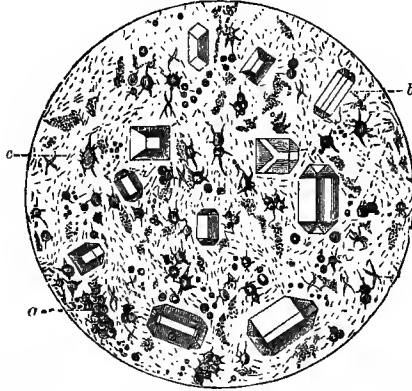


FIG. 86.—DEPOSIT IN AMMONIACAL FERMENTATION.  
*a*, Acid ammonium urate; *b*, ammonium magnesium phosphate; *c*, bacteria.

Occasionally a urine which possesses a normal acidity when voided, upon standing instead of undergoing ammoniacal fermentation as above described will become still more strongly acid in reaction. Such a phenomenon is termed *acid fermentation*. Accompanying this increased

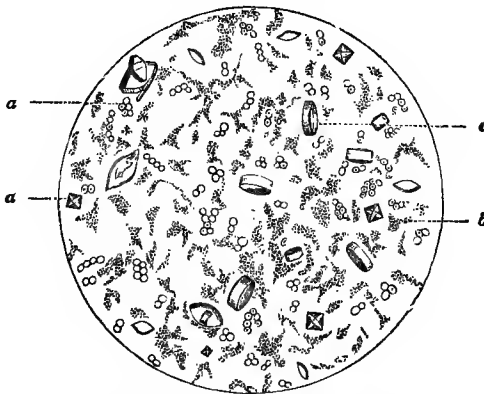


FIG. 87.—DEPOSIT IN ACID FERMENTATION.  
*a*, Fungus; *b*, amorphous sodium urate; *c*, uric acid; *d*, calcium oxalate.

acidity there is ordinarily a deepening of the tint of the urinary color. Such urines may contain *acid urates*, *uric acid*, *fungi*, and *calcium oxalate* (Fig. 87, above). On standing for a sufficiently long time any urine which exhibits acid fermentation will ultimately change in reaction,

due to the inception of alkaline fermentation, and will show the microscopical deposits characteristic of such a urine.

**Specific Gravity.**—The specific gravity of the urine of normal individuals varies ordinarily between 1.015 and 1.025. This value is subject to wide fluctuations under various conditions. For instance, following copious water- or beer-drinking the specific gravity may fall to 1.003 or lower, whereas in cases of excessive perspiration it may rise as high as 1.040 or even higher. Where a very accurate determination

of the specific gravity is desired use is commonly made of the *pyknometer* or of the *Westphal hydrostatic balance*. These instruments, however, are not suited for clinical use. The clinical method of determining the specific gravity is by means of a *urinometer* (Fig. 88). This affords a very rapid method and at the same time is sufficiently accurate for clinical purposes. The urinometer is always calibrated for use at a specific temperature and the observations made at any other temperature must be subjected to a certain correction to obtain the true specific gravity. In making this correction *one unit of the last order is added* to the observed specific gravity for every three degrees *above* the normal temperature and *subtracted* for every three degrees *below* the normal temperature. For instance, if in using a urinometer calibrated for 15° C. the specific gravity of a urine having a temperature of 21° C. is determined as 1.018 it is necessary to add to the observed specific gravity two units of the third order to obtain the real specific gravity of the urine. Therefore the true specific gravity, at 15° C., of a urine having a specific gravity of 1.018 at 21° C. is  $1.018 + 0.002 = 1.020$ .

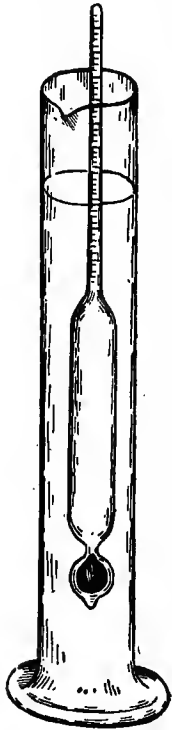


FIG. 88.—URINOMETER AND CYLINDER.

Pathologically, the specific gravity may be subjected to very wide variations. This is especially true in diseases of the kidneys. In acute nephritis ordinarily the urine is concentrated and of a high specific gravity, whereas in chronic nephritis the reverse conditions are more apt to prevail. In fact, under most conditions, whether physiological or pathological, the specific gravity of the urine is inversely proportional to the volume excreted. This is not true of diabetes mellitus, however, where the volume of urine is large and the specific gravity is also high, owing to the sugar contained in the urine.

The amount of solids eliminated in the excretion for twenty-four hours may be roughly calculated by means of *Long's coefficient*; i. e., 2.6.

The solid content of 1000 c.c. of urine is obtained by multiplying the last two figures of the specific gravity observed at 25° C. by 2.6. To determine the amount of solids excreted in twenty-four hours if the volume was 1120 c.c. and the specific gravity was 1.018 the calculation would be as follows:

$$(a) 18 \times 2.6 = 46.8 \text{ grams of solid matter in 1000 c.c. of urine.}$$

$$(b) \frac{46.8 \times 1120}{1000} = 52.4 \text{ grams of solid matter in 1120 c.c. of urine.}$$

The coefficient of Häser (2.33) which has been in use for years probably gives values that are inaccurate for conditions existing in America. This coefficient was calculated on the basis of the specific gravity determined at a temperature of 15° C.

**Freezing-point (Cryoscopy).**—The freezing-point of a solution depends upon the total number of molecules of solid matter dissolved in it. The determination of the osmotic pressure by this method has recently come to be of some clinical importance, particularly as an aid in the diagnosis of kidney disorders. In this connection it is best to collect the urine from each kidney separately and determine the freezing-point in the individual samples so collected. By this means considerable aid in the diagnosis of renal diseases may be secured. The fluids most frequently examined cryoscopically are the blood (see p. 194) and the urine. The freezing-point is denoted by  $\Delta$ . The value of  $\Delta$  for normal urine varies ordinarily between  $-1.3^\circ$  and  $-2.3^\circ$  C., the freezing-point of pure water being taken as  $0^\circ$ .  $\Delta$  is subject to very wide fluctuations under unusual conditions. For instance, following copious water- or beer-drinking  $\Delta$  may have as high a value as  $-0.2^\circ$  C., whereas on a diet containing much salt and deficient in fluids the value of  $\Delta$  may be lowered to  $-3^\circ$  C. or even lower. The freezing-point of normal blood is generally about  $-0.56^\circ$  C. and is not subject to the wide variations noted in the urine, because of the tendency of the organism to maintain the normal osmotic pressure of the blood under all conditions. Variations between  $-0.51^\circ$  and  $-0.62^\circ$  C. may be due entirely to dietary conditions, but if any marked variation is noted it can, in most cases, be traced to a disordered kidney function.

Freezing-point determinations may be made by means of the Beckmann-Heidenhain apparatus (Fig. 89) or the Zikel pektoscope. The Beckmann-Heidenhain apparatus consists of the following parts: A strong battery jar or beaker (C) furnished with a metal cover which is provided with a circular hole in its center. This strong glass vessel serves to hold the freezing mixture by means of which the temperature of the fluid under examination is lowered. A large glass tube (B) designed as an air-jacket, and formed after the manner of a test-tube is

introduced through the central aperture in the metal cover and into this air-jacket is lowered a smaller tube (A) containing the fluid to be tested. A very delicate thermometer (D), graduated in hundredths of a degree

is introduced into the inner tube and is held in place by means of a cork so that the mercury bulb is immersed in the fluid under examination but does not come in contact with any glass surface. A small platinum wire stirrer serves to keep the fluid under examination well mixed while a larger stirrer is used to manipulate the freezing mixture. (Rock salt and ice in the proportion 1:3 form a very satisfactory freezing mixture.)

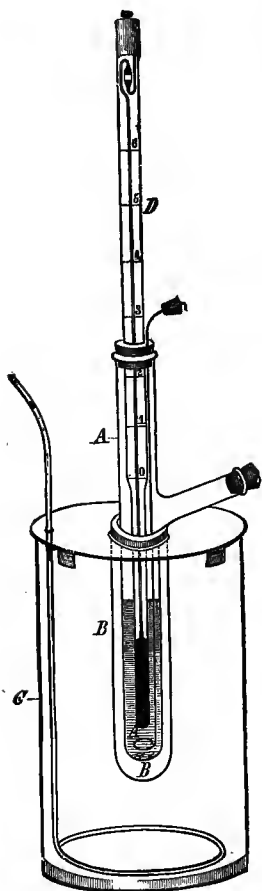


FIG. 89.—BECKMANN-HEIDENHAIN FREEZING-POINT APPARATUS. (Long.)

D, a delicate thermometer; C, the containing jar; B, the outside or air mantle tube; A, the tube in which the mixture to be observed is placed. Two stirrers are shown, one for the cooling mixture in the jar and one for the experimental mixture.

In making a determination of the freezing-point of a fluid by means of the Beckmann-Heidenhain apparatus proceed as follows: Place the freezing mixture in the battery jar and add water (if necessary) to secure a temperature not lower than  $3^{\circ}$  C. Introduce the fluid to be tested into tube A, place the thermometer and platinum wire stirrer in position, and insert the tube into the air-jacket which has previously been inserted through the metal cover of the battery jar. Manipulate the two stirrers in order to insure an equalization of temperature and observe the course of the mercury column of the thermometer very carefully. The mercury will *gradually fall* and this gradual lowering of the temperature will be followed by a *sudden rise*. The point at which the mercury rests *after this sudden rise* is the *freezing-point*. This rise is due to the fact that previous to freezing, a fluid is always more or less *over-cooled* and the thermometer temporarily registers a temperature somewhat *below the freezing-point*. As the fluid freezes, however, there is a very *sudden* change in the temperature of the liquid and this change is imparted to the

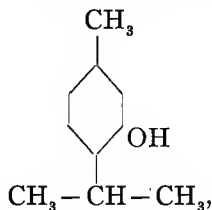
thermometer and causes the rise as indicated. It occasionally occurs that the fluid under examination is very much *over-cooled* and *does not freeze*. Under such circumstances a small piece of ice is introduced into it by means of the side tube noted in the figure. This so-called "inocula-



tion" causes the fluid to freeze instantaneously. (For details of the method of determining the freezing-point consult standard works on physical or organic chemistry.)

**Electrical Conductivity.**—The electrical conductivity of the urine is dependent upon the number of *inorganic* molecules or ions present, and in this differs from the freezing-point which is dependent upon the total number of molecules both *inorganic* and *organic* which are in solution. The conductivity of the urine has been investigated but slightly, and this rather recently, but from the data secured it seems that the value generally falls below  $\kappa=0.03$ . The conductivity of blood serum has been determined as  $\kappa=0.012$ . Up to the present time the determination of the electrical conductivity of any of the fluids of the body has been put to very slight clinical use. Experience may show the conductivity value to be a more important aid to diagnosis than it is now considered, particularly if it is taken in connection with the determination of the freezing-point. By a combination of these two methods the portion of the osmotic pressure due respectively to electrolytes and non-electrolytes may be determined. For a discussion of electrical conductivity, the method by which it is determined, and the principles involved consult standard works on physical or electro-chemistry.

**Collection of the Urine Sample.**—If any dependable data are desired regarding the *quantitative* composition of the urine the examination of the mixed excretion for twenty-four hours is *absolutely necessary*. In collecting the urine the bladder may be emptied at a given hour, say 8 A. M., the urine discarded and all the urine from that hour up to and including that passed the next day at 8 A. M., saved, thoroughly mixed, and a sample taken for analysis. Powdered thymol,



is a very satisfactory preservative since the excess may be removed by filtration, if desired, and any small amount which may go into solution will have no appreciable influence upon the determination of any of the urinary constituents. It has no reducing power and so may safely be used to preserve diabetic urines. To insure the preservation of the mixed urine of the twenty-four hour period it is advisable to place a small amount of the thymol powder in the urine receptacle before the first fraction of urine is voided. In order to further insure the preser-

vation of the urine the cleaned and dried urine receptacle may be rinsed with an alcoholic solution of thymol and subsequently thoroughly dried before introducing the urine.

Toluol is also used for the preservation of urine.

In certain pathological conditions it is desirable to collect the urine passed during the *day* separately from that passed during the *night*. When this is done the urine voided between 8 A. M. and 8 P. M. may be taken as the *day sample* and that voided between 8 P. M. and 8 A. M. as the *night sample*.

The *qualitative* testing of urine voided at *random*, except in a few specific instances, is of no particular value so far as giving us any accurate knowledge as to the exact urinary characteristics of the individual is concerned. In the great majority of cases the qualitative as well as the quantitative tests should be made upon the mixed excretion for a twenty-four hour period as well as upon a *night sample* as above described.

## CHAPTER XVIII.

### URINE : PHYSIOLOGICAL CONSTITUENTS.<sup>1</sup>

#### i. Organic Physiological Constituents.

Urea.	
Uric acid.	
Creatinine.	
Creatine. <sup>2</sup>	
Ethereal sulphuric acids . . . . .	{ Indoxyl-sulphuric acid. Phenol- and <i>p</i> -cresol-sulphuric acids. Pyrocatechin-sulphuric acid. Skatoxyl-sulphuric acid.
Hippuric acid.	
Oxalic acid.	
Neutral sulphur compounds . . . . .	{ Cystine. Chondroitin-sulphuric acid. Thiocyanates. Taurine derivatives. Oxyproteic acid. Alloxyproteic acid. Uroferric acid.
Allantoin.	
Aromatic oxyacids . . . . .	{ Paraoxyphenyl-acetic acid. Paraoxyphenyl-propionic acid. Homogentisic acid. Uroleucic acid. Oxymandelic acid. Kynurenic acid.
Amino acids.	
Benzoic acid.	
Neucleoprotein.	
Oxaluric acid.	

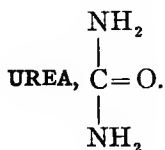
<sup>1</sup> It is impossible to make any *absolute* classification of the physiological and pathological constituents of the urine. A substance may be present in the urine in small amount physiologically and be sufficiently increased under certain conditions as to be termed a pathological constituent. Therefore it depends, in some instances, upon the *quantity* of a constituent present whether it may be correctly termed a physiological or a pathological constituent.

<sup>2</sup> Normal constituent of urine of infants and children (see p. 258).

Enzymes.....	{ Pepsin. Gastric rennin. Amylase.
Volatile fatty acids.....	{ Acetic acid. Butyric acid. Formic acid.
Paralactic acid.	
Phenaceturic acid.	
Phosphorized compounds.....	{ Glycerophosphoric acid. Phosphocarnic acid.
Pigments.....	{ Urochrome. Uroblin. Uroerythrin.
Ptomaines and leucomaines.	
Purine Bases.....	{ Adenine. Guanine. Xanthine. Epiguanine. Episarkine. Hypoxanthine. Paraxanthine. Heteroxanthine. 1-Methylxanthine.

## 2. Inorganic Physiological Constituents.

Ammonia.  
Sulphates.  
Chlorides.  
Phosphates.  
Sodium and potassium.  
Calcium and magnesium.  
Carbonates.  
Iron.  
Fluorides.  
Nitrates.  
Silicates.  
Hydrogen peroxide.



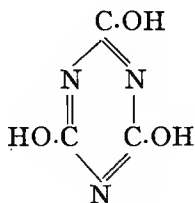


in amount after a diet of meat, and least in amount after a diet consisting of non-nitrogenous foods; this is due to the fact that the last-mentioned diet has a tendency to decrease the metabolism of the tissue proteins and thus cause the output of urea under these conditions to fall below the output of urea observed during starvation. The output of urea is also increased after copious water- or beer-drinking. The increase is probably due *primarily* to the washing out of the tissues of the urea previously formed, but which had not been removed in the normal processes, and *secondarily* to a stimulation of protein catabolism.

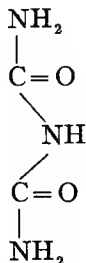
Urea may be formed in the organism from amino acids such as leucine, glycocoll, and aspartic acid: it may also be formed from ammonium carbonate  $(\text{NH}_4)_2\text{CO}_3$  or ammonium carbamate,  $\text{H}_4\text{N.O.CO.NH}_2$ .

There are differences of opinion regarding the transformation of the substances just named into urea, but there is rather conclusive evidence that at least a part of the urea is formed in the liver; it may be formed in other organs or tissues as well.

Urea crystallizes in long, colorless, four- or six-sided, anhydrous, rhombic prisms (Fig. 90, p. 285), which melt at  $132^\circ \text{C}$ . and are soluble in water or alcohol and insoluble in ether or chloroform. If a crystal of urea is heated in a test-tube, it melts and decomposes with the liberation of ammonia. The residue contains *cyanuric acid*,

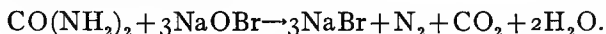


and *biuret*,



The biuret may be dissolved in water and a reddish-violet color obtained by treating the aqueous solution with copper sulphate and potassium hydroxide (see Biuret Test, p. 98). Certain hypochlorites or hypobromites in alkaline solution have the power of decomposing urea into

nitrogen, carbon dioxide, and water. Sodium hypobromite brings about this decomposition, as follows:



This property forms the basis for a clinical quantitative determination of urea (see page 392).

Urea has the power of forming crystalline compounds with certain acids; urea nitrate and urea oxalate are the most important of these compounds. *Urea nitrate*,  $\text{CO}(\text{NH}_2)_2 \cdot \text{HNO}_3$ , crystallizes in colorless, rhombic or six-sided tiles (Fig. 91, below), which are easily soluble in water. *Urea oxalate*,  $2 \cdot \text{CO}(\text{NH}_2)_2 \cdot \text{H}_2\text{C}_2\text{O}_4$ , crystallizes in the form of rhombic or six-sided prisms or plates (Fig. 93, p. 289): the oxalate differs from the nitrate in being somewhat less soluble in water.

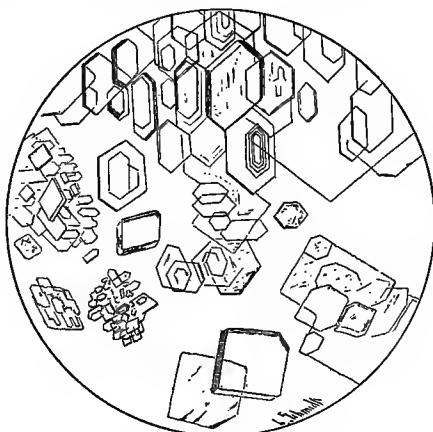


FIG. 91.—UREA NITRATE.

A decrease in the excretion of urea is observed in many diseases in which the diet is much reduced and in some disorders as a result of alterations in metabolism, *e. g.*, myxœdema, and in others as a result of changes in excretion, as in severe and advanced kidney disease. A pathological increase is found in a large proportion of diseases which are associated with a toxic state.

#### EXPERIMENTS ON UREA.

1. **Isolation from the Urine.**<sup>1</sup>—Place 800 c.c. of urine in a precipitating jar, add 250 c.c. of baryta mixture,<sup>2</sup> and stir thoroughly.

<sup>1</sup> The method based upon the precipitation by nitric acid is also satisfactory (see Hoppe-Seyler's *Handbuch der Physiol. und Pathol. Chem. Anal.*, Eighth edition, 1909, p. 145.)

<sup>2</sup> Baryta mixture consists of a mixture of one volume of a saturated solution of  $\text{Ba}(\text{NO}_3)_2$  and two volumes of a saturated solution of  $\text{Ba}(\text{OH})_2$ .

Filter off the precipitate of phosphates, sulphates, urates, and hippurates and evaporate the filtrate on a water-bath to a thick syrup. This syrup contains chlorides, creatinine, organic salts, pigments, and urea. Extract the syrup with warm 95 per cent alcohol and filter again. The filtrate contains the urea contaminated with pigment. Decolorize the filtrate by boiling with animal charcoal, filter again, and stand the filtrate away in a cold place for crystallization. Examine the crystals under the microscope and compare them with those shown in Fig. 90, page 285.

2. **Solubility.**—Test the solubility of urea, prepared by yourself or furnished by the instructor, in the ordinary solvents (see p. 27) and in alcohol and ether.

3. **Melting-point.**—Determine the melting-point of some pure urea furnished by the instructor. Proceed as follows: Into an ordinary melting-point tube, sealed at one end, introduce a crystal of urea. Fasten the tube to the bulb of a thermometer as shown in Fig. 92, and suspend the bulb and its attached tube in a small beaker containing sulphuric acid. Gently raise the temperature of the acid by means of a low flame, stirring the fluid continually, and note the temperature at which the urea begins to melt.

4. **Crystalline Form.**—Dissolve a crystal of pure urea in a few drops of 95 per cent alcohol and place 1–2 drops of the alcoholic solution on a microscopic slide. Allow the alcohol to evaporate spontaneously, examine the crystals under the microscope, and compare them with those reproduced in Fig. 90, p. 285. Recrystallize a little urea from water in the same way and compare the crystals with those obtained from the alcoholic solution.

5. **Formation of Biuret.**—Place a small amount of urea in a *dry* test-tube and heat carefully in a low flame. The urea melts at  $132^{\circ}$  C. and liberates ammonia. Continue heating until the fused mass begins to solidify. Cool the tube, dissolve the residue in dilute potassium hydroxide solution, and add very dilute copper sulphate solution (see p. 98). The purplish-violet color is due to the presence of biuret which has been formed from the urea through the application of heat as indicated. This is the reaction:

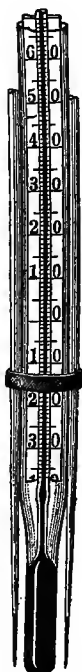
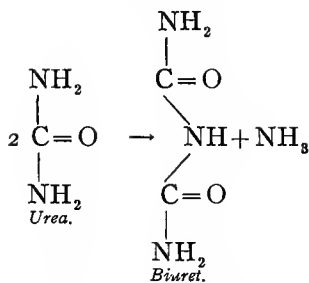


FIG. 92.—MELTING-POINT TUBES FASTENED TO BULB OF THERMOMETER.





6. **Urea Nitrate.**—Prepare a concentrated solution of urea by dissolving a little of the substance in a few drops of water. Place a drop of this solution on a microscopic slide, add a drop of concentrated nitric acid, and examine under the microscope. Compare the crystals with those reproduced in Fig. 91, p. 287.

7. **Urea Oxalate.**—To a drop of a concentrated solution of urea, prepared as described in the last experiment (6), add a drop of a satu-

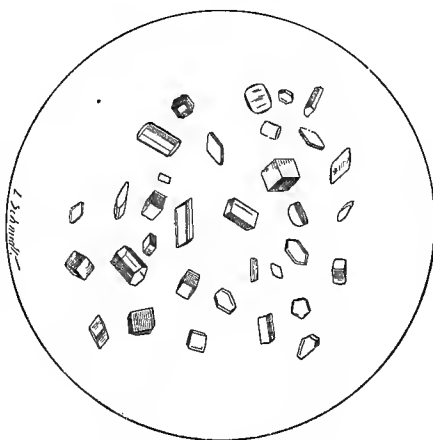


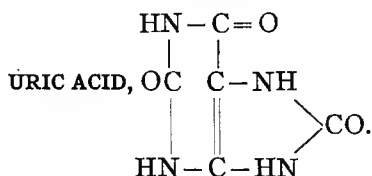
FIG. 93.—UREA OXALATE.

rated solution of oxalic acid. Examine under the microscope and compare the crystals with those shown in Fig. 93, above.

8. **Decomposition by Sodium Hypobromite.**—Into a mixture of 3 c.c. of concentrated sodium hydroxide solution and 2 c.c. of bromine water in a test-tube introduce a crystal of urea or a small amount of concentrated solution of urea. Through the influence of the sodium hypobromite,  $\text{NaOBr}$ , the urea is decomposed and carbon dioxide and nitrogen are liberated. The carbon dioxide is absorbed by the excess of sodium hydroxide, while the nitrogen is evolved and causes the marked effervescence observed. This property forms the basis for one of the methods in common use for the quantitative determination of urea. Write the

equation showing the decomposition of urea by sodium hypobromite.

9. **Furfurol Test.**—To a few crystals of urea in a small porcelain dish add 1–2 drops of a concentrated aqueous solution of furfural and 1–2 drops of concentrated hydrochloric acid. Note the appearance of a yellow color which gradually changes into a purple. Allantoin also responds to this test (see page 305).



Uric acid is one of the most important of the constituents of the urine. It is generally stated that normally about 0.7 gram is excreted in 24 hours but that this amount is subject to wide variations, particularly under certain dietary and pathological conditions. Very recently it has been shown that the average daily excretion of uric acid for ten men ranging in age from 19 to 29 years and fed a normal mixed diet was 0.597 gram, a value somewhat lower than the generally accepted average of 0.7 gram for such a period. Uric acid is a diureide and consequently upon oxidation yields two molecules of urea. It acts as a weak dibasic acid and forms two classes of salts, neutral and acid. The neutral potassium and lithium urates are the most easily soluble of the alkali salts; the ammonium urate is difficultly soluble. The acid-alkali urates are more insoluble and form the major portion of the sediment which separates upon cooling the concentrated urine; the alkaline earth urates are very insoluble. Ordinarily uric acid occurs in the urine in the form of urates and upon acidifying the liquid the uric acid is liberated and deposits in crystalline form. This property forms the basis of one of the older methods for the quantitative determination of uric acid (Heintz Method, p. 390).

Uric acid is very closely related to the purine bases as may be seen from a comparison of its structural formula with those of the purine bases given on page 261. According to the purine nomenclature it is designated 2-6-8-trioxypurine. Uric acid forms the principal end-product of the nitrogenous metabolism of birds and scaly amphibians; in the human organism it occupies the fourth position inasmuch as here urea, ammonia, and creatinine are the chief end-products of nitrogenous metabolism. It is generally said that the relation existing between uric acid and urea in human urine under normal conditions varies on the average from 1:40 to 1:100 and is subject to wider variations under pathological conditions; and further that because of the high content of



PLATE V.



URIC ACID CRYSTALS. NORMAL COLOR. (From Purdy, after Peyer.)

uric acid in the urine of new-born infants the ratio may be reduced to 1:10 or even lower. We now know that this ratio of uric acid to urea is of little significance under any conditions.

In man, uric acid probably results principally from the destruction of nuclein material. It may arise from nuclein or other purine material ingested as food or from the disintegrating cellular matter of the organism. The uric acid resulting from the first process is said to be of *exogenous* origin, whereas the product of the second form of activity is said to be of *endogenous* origin. As a result of experimentation, Sivé, and Burian and Schur, and Rockwood claim that the amount of endogenous uric acid formed in any given period is fairly constant for each individual under normal conditions, and that it is entirely independent of the total amount of nitrogen eliminated. Recently Folin has taken exception to the statements of these investigators and claims that, following a pronounced decrease in the amount of protein metabolized, the absolute quantity of uric acid is decreased but that this decrease is relatively smaller than the decrease in the total nitrogen excretion and that the per cent of the uric acid nitrogen, in terms of the total nitrogen, is therefore decidedly increased.

In birds and scaly amphibians the formation of uric acid is analogous to the formation of urea in man. In these organisms it is derived principally from the protein material of the tissues and the food and is formed through a process of synthesis which occurs for the most part in the liver; a comparatively small fraction of the total uric acid excretion of birds and scaly amphibians may result from nuclein material.

When pure, uric acid may be obtained as a white, odorless, and tasteless powder, which is composed principally of small, transparent, crystalline, rhombic plates. Uric acid as it separates from the urine is invariably pigmented, and crystallizes in a large variety of characteristic forms, *e. g.*, dumb-bells, wedges, rhombic prisms, irregular rectangular or hexagonal plates, whetstones, prismatic rosettes, etc. Uric acid is insoluble in alcohol and ether, soluble with difficulty in boiling water (1:1800) and practically insoluble in cold water (1:39,480, at 18° C.). It is soluble in alkalis, alkali carbonates, boiling glycerol, concentrated sulphuric acid, and in certain organic bases such as ethylamine and piperidine. It is claimed that the uric acid is held in solution in the urine by the urea and disodium hydrogen phosphate present. Uric acid possesses the power of reducing cupric hydroxide in alkaline solution and may thus lead to an erroneous conclusion in testing for sugar in the urine by means of Fehling's or Trommer's tests. A white precipitate of cuprous urate is formed if only a small amount of cupric hydroxide is present, but if enough of the copper salt is present the

characteristic red or brownish-red precipitate of cuprous oxide is obtained. Uric acid does not possess the power of reducing bismuth in alkaline solution and therefore does not interfere in testing for sugar in the urine by means of Boettger's or Nylander's tests.

In addition to being an important urinary constituent uric acid is normally present in the brain, heart, liver, lungs, pancreas, and spleen; it also occurs in the blood of birds and has been detected in traces in human blood under normal conditions.

Pathologically, the excretion of uric acid is subject to wide variations, but the experimental findings are rather contradictory. It may be stated with certainty, however, that in leukæmia the uric acid output is increased absolutely as well as relatively to the urea output; under these conditions the ratio between the uric acid and urea may be as low as 1:9, whereas the normal ratio, as we have seen, is 1:50 or higher. In the study of the influence of X-ray on metabolism Edsall reached some interesting conclusions. He found that the excretion of uric acid is usually increased and that in some conditions, particularly in leukæmia, it may be *greatly* increased. The excretion of total nitrogen, phosphates, and other substances may also be considerably increased.

#### EXPERIMENTS ON URIC ACID.

1. **Isolation from the Urine.**—Place about 200 c.c. of filtered urine in a beaker, render it acid with 2–10 c.c. of concentrated hydrochloric acid, stir thoroughly, and stand the vessel in a cold place for 24 hours. Examine the pigmented crystals of uric acid under the microscope and compare them with those shown in Fig. 106, p. 365 and Pl. V., opposite p. 291.

2. **Solubility.**—Try the solubility of pure uric acid, furnished by the instructor, in the ordinary solvents (see p. 27) and in alcohol, ether, concentrated sulphuric acid and in boiling glycerol.

3. **Crystalline Form of Pure Uric Acid.**—Place about 100 c.c. of water in a small beaker, render it distinctly alkaline with potassium hydroxide solution and add a small amount of pure uric acid, stirring continuously. Cool the solution, render it distinctly acid with hydrochloric acid and allow it to stand in a cool place for crystallization. Examine the crystals under the microscope and compare them with those reproduced in Fig. 94, p. 293.

4. **Murexide Test.**—To a small amount of pure uric acid in a small evaporating dish add 2–3 drops of concentrated nitric acid. Evaporate to dryness carefully on a water-bath or over a very low flame. A red or yellow residue remains which turns purplish-red after cooling the

dish and adding a drop of very dilute ammonium hydroxide. The color is due to the formation of *murexide*. If potassium hydroxide is used instead of ammonium hydroxide a purplish-violet color due to the production of the potassium salt is obtained. The color disappears upon warming; with certain related bodies (purine bases) the color persists under these conditions.

5. **Moreigne's Reaction.**—To equal volumes of Moreigne's reagent<sup>1</sup> and the solution to be tested add a few drops of concentrated potassium hydroxide. A blue color indicates the presence of uric acid.

6. **Schiff's Reaction.**—Dissolve a small amount of pure uric acid in sodium carbonate solution and transfer a drop of the resulting mix-

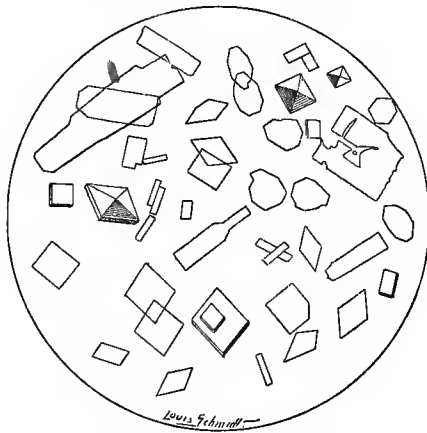


FIG. 94.—PURE URIC ACID.

ture to a strip of filter paper saturated with silver nitrate solution. A yellowish-brown or black coloration due to the formation of reduced silver is produced.

7. **Ganassini's Test.**<sup>2</sup>—Dissolve a small amount of uric acid in sodium carbonate. Precipitate the dissolved uric acid by means of zinc chloride, filter off the precipitate, and permit it to stand in contact with the air. A sky-blue color will develop, a color change which may be hastened by sunlight. A similar reaction may be obtained by treating the original precipitate with  $K_2S_2O_8$ .

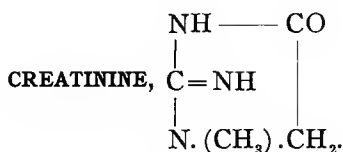
8. **Influence upon Fehling's Solution.**—Dilute 1 c.c. of Fehling's solution with 4 c.c. of water and heat to boiling. Now add *slowly*, a few drops at a time, 1–2 c.c. of a concentrated solution of uric acid in

<sup>1</sup> Moreigne's reagent is made by combining 20 grams of sodium tungstate, 10 grams of phosphoric acid (sp. gr. 1.13) and 100 c.c. of water. Boil this mixture for twenty minutes, add water to make the volume of the solution equivalent to the original volume, and acidify with hydrochloric acid.

<sup>2</sup> Ganassini: *Boll. soc.*, 1908, No. 1.

potassium hydroxide, heating after each addition. From this experiment what do you conclude regarding the possibility of arriving at an erroneous decision when testing for sugar in the urine by means of Fehling's test?

9. **Reduction of Nylander's Reagent.**—To 5 c.c. of a solution of uric acid in potassium hydroxide add about one-half a cubic centimeter of Nylander's reagent and heat to boiling for a few moments. Do you obtain the typical black end-reaction signifying the reduction of the bismuth?



Creatinine is the anhydride of creatine and is a constituent of normal human urine. The theory that creatinine is derived from the creatine of ingested muscular tissue as well as from the creatine of the muscular tissue of the organism has recently been proven to be incorrect by Folin, Klercker, and Wolf and Shaffer. Shaffer believes that creatinine is the result of some special process of normal metabolism which takes place to a large extent, if not entirely, in the muscles and further that the amount of such creatinine elimination, expressed *in milligrams per kilogram body weight*, is an index of this special process.<sup>1</sup> He further states that the muscular efficiency of the individual depends upon the intensity of this process. Under normal conditions about 1 gram of creatinine is excreted by an adult man in 24 hours,<sup>2</sup> the exact amount depending in great part upon the nature of the food and decreasing markedly in starvation. Very little that is important is known regarding the excretion of creatinine under pathological conditions. The creatinine content of the urine is said to be increased in typhoid fever, typhus, tetanus, and pneumonia, and to be decreased in anæmia, chlorosis, paralysis, muscular atrophy, advanced degeneration of the kidneys, and in leukæmia (myelogenous, lymphatic and pseudo). An increase of creatinine was also noted in diabetes, an increase probably due to the creatinine content of the meat eaten. The greater part of the data, however, relating to the variation of the creatinine excretion under pathological conditions are not of much value since in nearly every instance the diet was not sufficiently controlled to permit the collection of reliable data. And further, until the advent of the Folin method (see p. 415), there was no accurate method for the quantitative determina-

<sup>1</sup> He proposes to designate as the "creatinine coefficient" the excretion of *creatinine-nitrogen* (*mgs.*) *per kilogram of body weight*.

<sup>2</sup> According to Shaffer the amount excreted by strictly normal individuals is between 7 and 11 milligrams of creatinine-nitrogen per kilogram of body weight.



tion of creatinine. Shaffer has very recently called attention to the fact that a low excretion of creatinine is found in the urine of a remarkably large number of pathological subjects, representing a variety of conditions, and that it is therefore evident that the excretion of an abnormally small amount of this substance is by no means peculiar to any one disease.

Creatinine crystallizes in colorless, glistening monoclinic prisms (Fig. 95, below) which are soluble in about 12 parts of cold water; they are more soluble in warm water and in warm alcohol. It forms salts only with strong mineral acids. One of the most important and interesting of the compounds of creatinine is *creatinine-zinc chloride*,  $(C_4H_7N_3O)_2 \cdot ZnCl_2$ , which is formed from an alcoholic solution of creatinine upon



FIG. 95.—CREATININE.

treatment with zinc chloride in acid solution. Creatinine has the power of reducing cupric hydroxide in alkaline solution and in this way may interfere with the determination of sugar in the urine. In the reduction by creatinine the blue liquid is first changed to a yellow and the formation of a brownish-red precipitate of cuprous oxide is brought about only after continuous boiling with an excess of the copper salt. Creatinine does not reduce alkaline bismuth solutions and therefore does not interfere with Nylander's and Boettger's tests.

It has recently been shown by Folin that the absolute quantity of creatinine eliminated in the urine on a meat-free diet is a constant quantity different for different individuals, but wholly independent of quantitative changes in the total amount of nitrogen eliminated. Shaffer has very recently confirmed these findings and has shown that the output of creatinine under these conditions is constant from hour to hour as well as from day to day.

According to Pekelharing<sup>1</sup> muscular *tonus* increases the creatinine excretion of the individual whereas muscular *exertion* does not.

#### EXPERIMENTS ON CREATININE.

1. **Separation from the Urine.**—Place 250 c.c. of urine in a casserole or beaker, render it alkaline with milk of lime and then add  $\text{CaCl}_2$  solution until the phosphates are completely precipitated. Filter off the precipitate, render the filtrate slightly acid with acetic acid, and evaporate it to a syrup. While still warm this syrup is treated with about 50 c.c. of 95–97 per cent alcohol and the mixture allowed to stand 8–12 hours in a cool place. The precipitate is now filtered off and the filtrate treated with a little sodium acetate and about one-half c.c. of acid-free zinc chloride solution having a specific gravity of 1.2. This mixture is stirred thoroughly

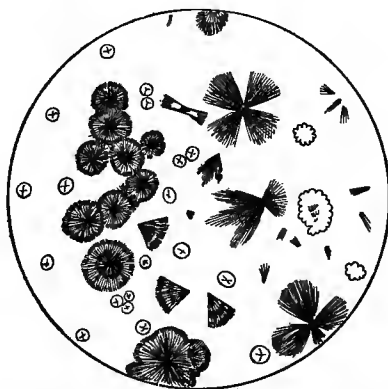


FIG. 96.—CREATININE-ZINC CHLORIDE. (Salkowski.)

and allowed to stand in a cold place for 48–72 hours. Creatinine-zinc chloride (Fig. 96, above) will crystallize out under these conditions. Collect the crystals on a filter paper and wash them with alcohol to remove chlorides. Now treat the zinc chloride compound with a little warm water, boil with lead oxide and filter. The filtrate may now be decolorized by animal charcoal, evaporated to dryness, and the residue extracted with strong alcohol. (Creatine remains undissolved under these conditions.) The alcoholic extract of creatinine is now evaporated to incipient crystallization and left in a cool place until crystallization is complete. If desired the crystals may be purified by recrystallization from water.

2. **Weyl's Test.**—Take 5 c.c. of urine in a test-tube, add a few drops of sodium nitro-prusside and render the solution alkaline with

<sup>1</sup> Pekelharing: *Onderzoekingen gedaan in het Physiol. Lab. te Utrecht*, Vol. 5, No. 12, 1911.

potassium hydroxide solution. A ruby-red color results which soon turns yellow. See Legal's test for acetone, page 349.

3. **Salkowski's Test.**—To the yellow solution obtained in Weyl's test above add an excess of acetic acid and apply heat. A green color results and is in turn displaced by a blue color. A precipitate of Prussian blue may form.

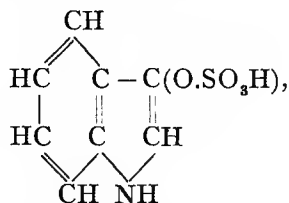
4. **Jaffe's Reaction.**—Place 5 c.c. of urine in a test-tube, add an aqueous solution of picric acid and render the mixture alkaline with potassium hydroxide solution. A red color is produced which turns yellow if the solution be acidified. Dextrose gives a similar red color but only upon the application of heat. This color reaction observed when creatinine in alkaline solution is treated with picric acid is the basic principle of Folin's colorimetric method for the quantitative determination of creatinine (see page 415.)

#### ETHEREAL SULPHURIC ACIDS.

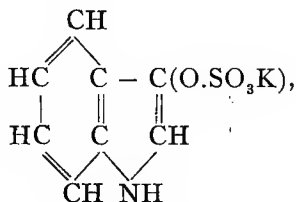
The most important of the ethereal sulphuric acids found in the urine are *phenol-sulphuric acid*, *p-cresol-sulphuric acid*, *indoxyl-sulphuric acid*, and *skatoxyl-sulphuric acid*. Pyrocatechin-sulphuric acid also occurs in traces in human urine. The total output of ethereal sulphuric acid varies from 0.09 to 0.62 gram for 24 hours. In health the ratio of ethereal sulphuric acid to inorganic sulphuric acid is about 1:10. These ethereal sulphuric acids originate in part from the phenol, cresol, indole and skatole formed in the putrefaction of protein material in the intestine. The phenol passes to the liver where it is conjugated to form phenol potassium sulphate and appears in this form in the urine whereas the indole and skatole undergo a preliminary oxidation to form *indoxyl* and *skatoxyl* respectively before their elimination.

It has generally been considered that each of the ethereal sulphuric acids was formed principally in the putrefaction of protein material in the intestine and that therefore a determination of the total ethereal sulphuric acid content of the urine was an index of the extent to which these putrefactive processes were proceeding within the organism. Recently, however, Folin has conducted a series of experiments which seem to show that the ethereal sulphuric acid content of the urine does *not* afford an index of the extent of intestinal putrefaction, since these bodies arise only in part from putrefactive processes. He claims that the ethereal sulphuric acid excretion represents a form of sulphur metabolism which is more in evidence upon a diet containing a very small amount of protein or upon a diet containing absolutely no protein. The ethereal sulphuric acid content of the urine diminishes as the total sulphur content diminishes but the *percentage decrease* is much less. Therefore when

considered from the standpoint of the total sulphuric acid content the ethereal sulphuric acid content is not diminished but is *increased*, although the total sulphuric acid content *is diminished*. Folin's experiments also seem to show that the indoxyl sulphuric acid (indoxyl potassium sulphate or indican) content of the urine does not originate to any degree from the metabolism of protein material but that it arises in great part from intestinal putrefaction and that the excretion of indoxyl sulphuric acid may *alone* be taken as a rough index of the extent of putrefactive processes within the intestine. Indoxyl sulphuric acid,



therefore, which occurs in the urine as indoxyl potassium sulphate or indican,



is clinically the most important of the ethereal sulphuric acids.

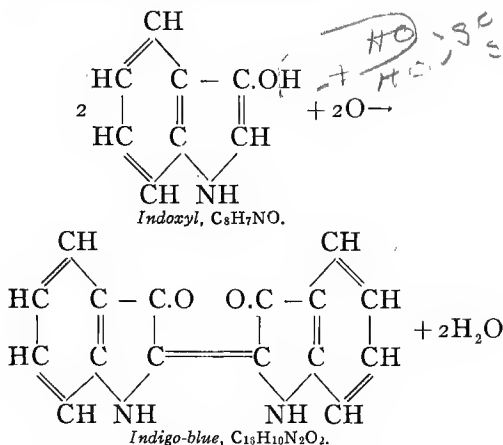
#### TESTS FOR INDICAN.<sup>1</sup>

1. **Jaffe's Test.**—Nearly fill a test-tube with a mixture composed of equal volumes of concentrated HCl and the urine under examination. Add 2–3 c.c. of chloroform and a few drops of a calcium hypochlorite solution, place the thumb over the end of the test-tube and shake the tube and contents thoroughly. The chloroform is colored more or less, according to the amount of indican present. Ordinarily a blue color due to the formation of indigo-blue is produced; less frequently a red color due to indigo-red may be noted.

Repeat this test on some of this same urine to which formaldehyde has been added. Is there any variation in the reaction from what you previously obtained?

<sup>1</sup> The urine should always be examined *fresh* if this is possible. In any event formaldehyde should never be used as a preservative for such urines as are to be examined for indican by means of any test involving hypochlorite or potassium permanganate. The formaldehyde through its reducing power lowers the oxidizing efficiency of the mixture. The formation of formic acid from the aldehyde may also interfere.

This is the reaction (see also page 169):



2. **Obermayer's Test.**—Nearly fill a test-tube with a mixture composed of equal volumes of Obermayer's reagent<sup>1</sup> and the urine under examination. Add 2–3 c.c. of chloroform, place the thumb over the end of the test-tube and shake thoroughly. How does this compare with Jaffe's test?

3. **Gürber's Reaction.**—To one volume of the urine under examination and two volumes of concentrated hydrochloric acid in a test-tube add 2–3 drops of a 1 per cent solution of osmic acid and 2–3 c.c. of chloroform and shake the tube and contents thoroughly. Compare the color with those obtained in Jaffe's and Obermayer's tests.

An excess of osmic acid does not affect the reaction. Occasionally better results are obtained if the solution of osmic acid is added directly to the urine *before* the addition of the hydrochloric acid. If the urine under examination be strongly colored or of high specific gravity it should first be treated with basic lead acetate (one-eighth volume). The precipitate is then removed by filtration and the resulting filtrate used in making the test for indican.

4. **Rossi's Reaction.**—To equal volumes of concentrated hydrochloric acid and the urine under examination, in a test-tube, add 1 drop of a 10 per cent solution of ammonium persulphate and 2–3 c.c. of chloroform. Agitate the mixture vigorously and note the color of the chloroform. Compare this result with those obtained in the other indican tests.

5. **Lavelle's Reaction.**—To 10 c.c. of urine in a test-tube add 2–3 c.c. of Obermayer's reagent<sup>1</sup> and a similar volume of concentrated

<sup>1</sup> Obermayer's reagent is prepared by adding 2–4 grams of ferric chloride to a liter of concentrated HCl (sp. gr. 1.19).

sulphuric acid. (During the addition of the acid the tube should be held under running water in order that the temperature of the mixture may not rise too high.) Add 2–3 c.c. of chloroform, shake the tube vigorously, and observe the depth of color assumed by the chloroform.

The sponsor for this reaction claims it to be the most satisfactory of the indican tests.

6. **Barberio's Reaction.**<sup>1</sup>—To 5 c.c. of the urine in a test-tube add 2–3 drops of a sodium nitrite solution (1:2000) and mix well by shaking. Now add 5 c.c. of concentrated hydrochloric acid and 2–3 c.c. of chloroform and again shake. Note the color of the chloroform. Compare this test with tests 1 and 2 on the same urine.



HIPPURIC ACID,



This acid occurs normally in the urine of both the carnivora and herbivora but is more abundant in the urine of the latter. It is formed



FIG. 97.—HIPPURIC ACID.

by a synthesis of benzoic acid and glycocholl which takes place in the kidneys. The average excretion of an adult man for 24 hours under normal conditions is about 0.7 gram. Hippuric acid crystallizes in needles or rhombic prisms (see Fig. 97, above) the particular form depending upon the rapidity of crystallization. Pure hippuric acid melts at 187° C. The most satisfactory method for the isolation of hippuric acid from the urine in crystalline form is that proposed by Roaf (see below). It is easily soluble in alcohol or hot water, and

<sup>1</sup> Barberio: Policlinico, No. 17, 1911.

only slightly soluble in ether. The output of hippuric acid is increased in diabetes owing probably to the ingestion of much protein and fruit. It is decreased in fevers and in certain kidney disorders where the synthetic activity of the renal cells is diminished. Hippuric acid may be determined quantitatively by means of Dakin's methods (see p. 406).

#### EXPERIMENTS ON HIPPURIC ACID.

1. **Separation from the Urine.** (a) *First Method.*—Render 500–1000 c.c. of urine of the horse or cow<sup>1</sup> alkaline with milk of lime, boil for a few moments and filter while hot. Concentrate the filtrate, over a burner, to a small volume. Cool the solution, acidify it strongly with concentrated hydrochloric acid and stand it in a cool place for 24 hours. Filter off the crystals of hippuric acid which have formed and wash them with a little cold water. Remove the crystals from the paper, dissolve them in a very small amount of hot water and percolate the hot solution through thoroughly washed animal charcoal, being careful to wash out the last portion of the hippuric acid solution with hot water. Filter, concentrate the filtrate to a small volume and stand it aside for crystallization. Examine the crystals under the microscope and compare them with those in Fig. 97, page 300. This method is not as satisfactory as Roaf's method (see below).

(b) *Roaf's Method.*—Place 500 c.c. of urine of the horse or cow<sup>1</sup> in a casserole or precipitating jar and add an equal volume of a saturated solution of ammonium sulphate<sup>2</sup> and 7.5 c.c. of concentrated sulphuric acid. Permit the mixture to stand for twenty-four hours and remove the crystals of hippuric acid by filtration. Purify the crystals by recrystallization according to the directions given above under First Method. Examine the crystals under the microscope and compare them with those given in Fig. 97, p. 300.

If sufficient urine is not available to permit the use of 500 c.c. a smaller volume may be used inasmuch as it is possible, by the above technique, to isolate hippuric acid in crystalline form from as small a volume as 25–50 c.c. of herbivorous urine. The greater the amount of ammonium sulphate added the more rapid the crystallization until at the saturation point the crystals of hippuric acid sometimes form in about *ten minutes*.

<sup>1</sup> If urine of the horse or cow is not available human urine may serve the purpose fully as well provided means are taken to increase its content of hippuric acid. This may be conveniently accomplished by ingesting 2 grams of ammonium benzoate at night. The fraction of urine passed in the morning will be found to have a high content of hippuric acid. The ammonium benzoate is in no way harmful. In case ammonium benzoate is not available sodium benzoate may be substituted.

<sup>2</sup> 125 grams of *solid* ammonium sulphate may be substituted.

2. **Melting-point.**—Determine the melting-point of the hippuric acid prepared in the above experiment (see p. 146).

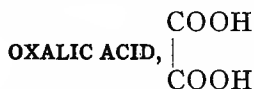
3. **Solubility.**—Test the solubility of hippuric acid in the ordinary solvents (page 27) and in alcohol, and ether.

4. **Dehn's Reaction.**—Introduce about 5 c.c. of the urine or the solution under examination into a test-tube and add sufficient hypobromite solution<sup>1</sup> to impart to the mixture a permanent yellow color. In the case of urine enough hypobromite should be added to decompose the urea. Heat the mixture to boiling and note the formation of an orange or brown-red precipitate if hippuric acid is present. If the solution under examination contains only a trace of hippuric acid the solution will appear smoky and faintly red in color, whereas if a larger amount of the acid be present the solution will become opaque and of an orange or brown-red color. In either case after standing for some time the solution should clear up and a light, finely divided precipitate should be deposited. This precipitate consists of earthy phosphates mixed with an amorphous orange or brown-red substance of unknown composition. (For some unknown reason this reaction does not always yield satisfactory results even on pure hippuric acid solution.)

5. **Formation of Nitro-Benzene.**—To a little hippuric acid in a small porcelain dish add 1-2 c.c. of concentrated HNO<sub>3</sub> and evaporate to dryness on a water-bath. Transfer the residue to a dry test-tube, apply heat, and note the odor of the artificial oil of bitter almonds (nitro-benzene).

6. **Sublimation.**—Place a few crystals of hippuric acid in a dry test-tube and apply heat. The crystals are reduced to an oily fluid which solidifies in a crystalline mass upon cooling. When stronger heat is applied the liquid assumes a red color and finally yields a sublimate of benzoic acid and the odor of hydrocyanic acid.

7. **Formation of Ferric Salt.**—Render a small amount of a solution of hippuric acid neutral with dilute potassium hydroxide. Now add 1-3 drops of neutral ferric chloride solution and note the formation of the ferric salt of hippuric acid as a cream colored precipitate.



Oxalic acid is a constituent of normal urine, about 0.02 gram being eliminated in 24 hours. It is present in the urine as calcium oxalate, which is kept in solution through the medium of the acid phosphates. The origin of the oxalic acid content of the urine is not well under-

<sup>1</sup> See note on p. 392.



tood. It is eliminated, at least in part, unchanged when ingested, therefore since many of the common articles of diet, *e. g.*, asparagus, apples, cabbage, grapes, lettuce, spinach, tomatoes, etc., contain oxalic acid it seems probable that the ingested food supplies a portion of the oxalic acid found in the urine. There is also experimental evidence that part of the oxalic acid of the urine is formed within the organism in the course of protein and fat metabolism. It has also been suggested that oxalic acid may arise from an incomplete combustion of carbohydrates, specially under certain abnormal conditions. Pathologically, oxalic acid is found to be increased in amount in diabetes mellitus, in organic diseases of the liver, and in various other conditions which are accompanied by a derangement of the oxidation mechanism. An abnormal increase of oxalic acid is termed *oxaluria*. A considerable increase in the content of oxalic acid may be noted unaccompanied by any other apparent symptom. Calcium oxalate crystallizes in at least two distinct forms, *dumb-bells* and *octahedra* (Fig. 104, page 363).

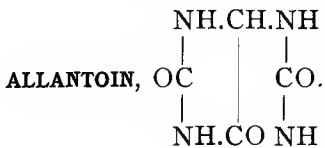
#### EXPERIMENTS.

**Preparation of Calcium Oxalate.** *First Method.*—Place 200–250 c.c. of urine in a beaker, add 5 c.c. of a saturated solution of calcium chloride, make the urine slightly acid with acetic acid, and stand the beaker aside in a cool place for 24 hours. Examine the sediment under the microscope and compare the crystalline forms with those shown in Fig. 104, p. 363.

*Second Method.*—Proceed as above, replacing the acetic acid by an excess of ammonium hydroxide and filtering off the precipitate of phosphates.

#### NEUTRAL SULPHUR COMPOUNDS.

Under this head may be classed such bodies as cystine (see p. 80), chondroitin-sulphuric acid, oxyproteic acid, alloxypoteic acid, uroferric acid, thiocyanates and taurine derivatives. The sulphur content of the bodies just enumerated is generally termed loosely combined or neutral sulphur in order that it may not be confused with the acid sulphur which occurs in the inorganic sulphuric acid and ethereal sulphuric acid forms. Ordinarily the neutral sulphur content of normal human urine is 14–20 per cent of the total sulphur content.



Allantoïn has been found in the urine of suckling calves as well as in that of the dog and cat, rabbit, monkey, horse and man.<sup>1</sup> It has also been detected in the urine of infants within the first eight days after birth, as well as in the urine of adults. It is more abundant in the urine of women during pregnancy. Underhill also reports the presence of allantoïn in the urine of fasting dogs, an observation which makes it probable that allantoïn is a constant constituent of the urine of such animals. Allantoïn is formed by the oxidation of uric acid and the output is increased by the feeding of thymus or pancreas to lower animals. In fact allantoïn is considered to be the principal end-product of purine



FIG. 98.—ALLANTOÏN, FROM CAT'S URINE.

*a* and *b*, Forms in which it crystallized from the urine; *c*, recrystallized allantoïn. (Drawn from micro-photographs furnished by Prof. Lafayette B. Mendel of Yale University.)

metabolism in such animals. Notwithstanding certain evidence<sup>2</sup> favoring this view it is not generally believed that allantoïn is an important end-product of purine metabolism in man. When pure it crystallizes in prisms (Fig. 98, above) and when impure in granules and knobs. Pathologically, it has been found increased in diabetes insipidus and in hysteria with convulsions (Pouchet). Mendel and Dakin<sup>3</sup> have recently shown that allantoïn is optically inactive notwithstanding the fact that it contains an asymmetric carbon atom. This phenomenon they believe to be due to tautomeric change. Wiechowski has suggested an excellent method for the quantitative determination of allantoïn.<sup>4</sup>

<sup>1</sup> Wiechowski: *Biochemische Zeitschrift*, 19, 368, 1909.

<sup>2</sup> Ascher: *Biochemische Zeitschrift*, 26, 370, 1910; Fairhall and Hawk: *Jour. Am. Chem. Soc.*, 34, 546, 1912.

<sup>3</sup> Mendel and Dakin: *Jour. Biol. Chem.*, 7, 153, 1910.

<sup>4</sup> Wiechowski: *Biochemische Zeitschrift*, 19, 368, 1909.

## EXPERIMENTS.

1. **Separation from the Urine.**<sup>1</sup> *Meissner's Method.*—Precipitate the urine with baryta water. Neutralize the filtrate *carefully* with dilute sulphuric acid, filter immediately, and evaporate the filtrate to incipient crystallization. Completely precipitate this *warm* fluid with 95 per cent alcohol (reserve the precipitate). Decant or filter and precipitate the solution by ether. Combine the ether and alcohol precipitates and extract with *cold* water or *hot* alcohol; allantoin remains undissolved. Bring the allantoin into solution in *hot* water and recrystallize.

Allantoin may be determined quantitatively by the Paduschka-Underhill-Kleiner method (see p. 432) or by Loewi's method.<sup>2</sup>

2. **Preparation from Uric Acid.**—Dissolve 4 grams of uric acid in 100 c.c. of water rendered alkaline with potassium hydroxide. Cool and *carefully* add 3 grams of potassium permanganate. Filter, *immediately* acidulate the filtrate with acetic acid and allow it to stand in a cool place over night. Filter off the crystals and wash them with water. Save the wash water and filtrate, unite them and after concentrating to a small volume stand away for crystallization. Now combine all the crystals and recrystallize them from hot water. Use these crystals in the experiments which follow.

3. **Microscopical Examination.**—Examine the crystals made in the last experiment and compare them with those shown in Fig. 98, page 304.

4. **Solubility.**—Test the solubility of allantoin in the ordinary solvents (page 27.)

5. **Reaction.**—Dissolve a crystal in water and test the reaction to litmus.

6. **Furfurol Test.**—Place a few crystals of allantoin on a test-tablet or in a porcelain dish and add 1-2 drops of a concentrated aqueous solution of furfurol and 1-2 drops of concentrated hydrochloric acid. Observe the formation of a yellow color which turns to a light purple if allowed to stand. This test is given by urea but not by uric acid.

7. **Murexide Test.**—Try this test according to the directions given on page 292. Note that allantoin fails to respond.

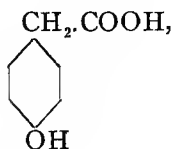
8. **Reduction of Fehling's Solution.**—Make this test in the usual way (see p. 32) except that the boiling must be prolonged and excessive. Ultimately the allantoin will reduce the solution. Compare with the result on uric acid, page 293.

<sup>1</sup> The urine of the dog after thymus, pancreas, or uric acid feeding may be employed.

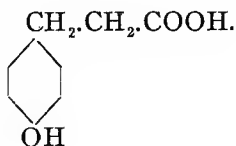
<sup>2</sup> Archiv für Experimentelle Pathologie und Pharmakologie, 44, 20, 1900.

## AROMATIC OXYACIDS.

Two of the most important of the oxyacids are *paraoxyphenyl-acetic acid*,

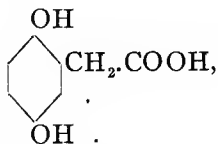


and *paraoxyphenyl-propionic acid*,



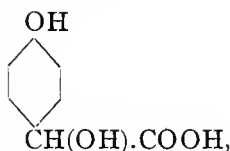
They are products of the putrefaction of protein material and tyrosine is an intermediate stage in their formation. Both these acids for the most part pass unchanged into the urine where they occur normally in very small amount. The content may be increased in the same manner as the phenol content, in particular by acute phosphorous poisoning. A fraction of the total aromatic oxyacid content of the urine is in combination with sulphuric acid, but the greater part is present in the form of salts of sodium and potassium.

*Homogentisic Acid* or di-oxyphenyl-acetic acid,



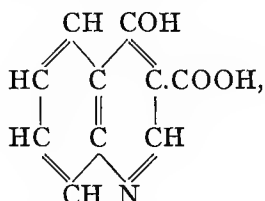
is another important oxyacid sometimes present in the urine. Under the name *glycosuric acid* it was first isolated from the urine by Prof. John Marshall of the University of Pennsylvania; subsequently Baumann isolated it and determined its chemical constitution. It occurs in cases of *alcaptonuria*. A urine containing this oxyacid turns greenish-brown from the surface downward when treated with a little sodium hydroxide or ammonia. If the solution be stirred the color very soon becomes dark brown or even black. Homogentisic acid reduces alkaline copper solutions but not alkaline bismuth solutions. Uroleucic acid is similar in its reactions to homogentisic acid.

*Oxymandelic Acid* or paraoxyphenyl-glycolic acid,



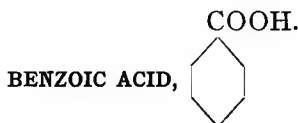
has been detected in the urine in cases of yellow atrophy of the liver.

*Kynurenic Acid* or  $\gamma$ -oxy- $\beta$ -quinoline carbonic acid,



is present in the urine of the dog and has recently been detected by Swain in the urine of the coyote. To isolate it from the urine proceed as follows: Acidify the urine with hydrochloric acid in the proportion 1:25. From this acid fluid both the uric acid and the kynurenic acid separate in the course of 24-48 hours. Filter off the combined crystalline deposit of the two acids, dissolve the kynurenic acid in dilute ammonia (uric acid is insoluble), and reprecipitate it with hydrochloric acid.

Kynurenic acid may be quantitatively determined by Capaldi's method.<sup>1</sup>



Benzoic acid has been detected in the urine of the rabbit and dog. It is also said to occur in human urine accompanying renal disorders. The benzoic acid probably originates from a fermentative decomposition of the hippuric acid of the urine.

#### EXPERIMENTS.

1. **Solubility.**—Test the solubility of benzoic acid in water, alcohol, and ether.

2. **Crystalline Form.**—Recrystallize some benzoic acid from hot water, examine the crystals under the microscope, and compare them with those reproduced in Fig. 99, p. 308.

3. **Sublimation.**—Place a little benzoic acid in a test-tube and heat

<sup>1</sup> *Zeitschrift für physiologische Chemie*, 23, 92, 1897.

over a flame. Note the odor which is evolved and observe that the acid sublimes in the form of needles.

4. Dissolve a little sodium benzoate in water and add a solution of neutral ferric chloride. Note the production of a brownish-yellow precipitate (salicylic acid gives a reddish-violet color under the same conditions). Add ammonium hydroxide to some of the precipitate. It dissolves and ferric hydroxide is formed. Add a little hydrochloric

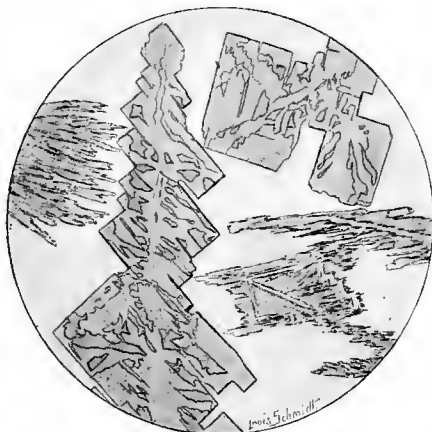
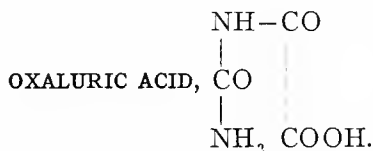


FIG. 99.—BENZOIC ACID.

acid to another portion of the original precipitate and stand the vessel away over night. What do you observe?

#### NUCLEOPROTEIN.

The nubecula of normal urine has been shown by one investigator to consist of a mucoïd containing 12.7 per cent of nitrogen and 2.3 per cent of sulphur. This body evidently originates in the urinary passages. It is probably slightly soluble in the urine. Some investigators believe that the body forming the nubecula of normal urine is nucleoprotein and not a mucin or mucoïd as stated above. A discussion of nucleoprotein and related bodies occurring in the urine under pathological conditions will be found on page 339.



Oxaluric acid is not a constant constituent of normal human urine, and when found occurs only in traces as the ammonium salt. Upon boiling oxaluric acid it splits into oxalic acid and urea.

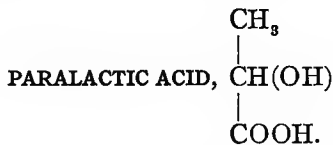
## ENZYMES.

Various types of enzymes produced within the organism are excreted in both the feces and the urine. In this connection it is interesting to note that *pepsin*, *gastric rennin*, and an *amylase* have been positively identified in the urine. The occurrence of *trypsin* in the urine, at least under normal conditions, is questioned.

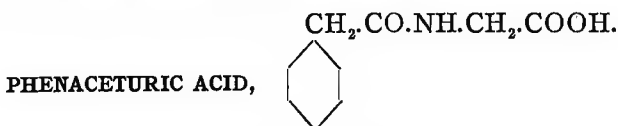
## VOLATILE FATTY ACIDS.

Acetic, butyric, and formic acids have been found under normal conditions in the urine of man and of certain carnivora as well as in the urine of herbivora. Normally they arise principally from the fermentation of carbohydrates and the putrefaction of proteins. The acids containing the fewest carbon atoms (formic and acetic) are found to be present in larger percentage than those which contain a larger number of such atoms. The volatile fatty acids occur in normal urine in traces, the total output for twenty-four hours, according to different investigators, varying from 0.008 gram to 0.05 gram.

Pathologically, the excretion of volatile fatty acids is increased in diabetes, fevers, and in certain hepatic diseases in which the parenchyma of the liver is seriously affected. Under other pathological conditions the output may be diminished. These variations, however, in the excretion of the volatile fatty acids possess very little diagnostic value.



Paralactic acid is supposed to pass into the urine when the supply of oxygen in the organism is diminished through any cause, *e. g.*, after acute yellow atrophy of the liver, acute phosphorus poisoning, or epileptic attacks. This acid has also been found in the urine of healthy persons following the physical exercise incident to prolonged marching. Paralactic acid has been detected in the urine of birds after the removal of the liver. Underhill reports the occurrence of this acid in the urine of a case of pernicious vomiting of pregnancy.



Phenaceturic acid occurs principally in the urine of herbivorous animals but has frequently been detected in human urine. It is pro-

duced in the organism through the synthesis of glycocholic and phenylacetic acid. It may be decomposed into its component parts by boiling with dilute mineral acids. The crystalline form of phenaceturic acid (small rhombic plates with rounded angles) resembles one form of uric acid crystal.

#### PHOSPHORIZED COMPOUNDS.

Phosphorus in organic combination has been found in the urine in such bodies as glycerophosphoric acid, which may arise from the decomposition of lecithin, and phosphocarnic acid. It is claimed that on the average about 2.5 per cent of the total phosphorus elimination is in organic combination.

#### PIGMENTS.

There are at least three pigments normally present in human urine. These pigments are *urochrome*, *urobilin*, and *uroerythrin*.

##### A. UROCHROME.

This is the principal pigment of normal urine and imparts the characteristic yellow color to that fluid. It is apparently closely related to its associated pigment urobilin since the latter may be readily converted into urochrome through evaporation of its aqueous-ether solution. Urochrome may be obtained in the form of a brown, amorphous powder which is readily soluble in water and 95 per cent alcohol. It is less soluble in absolute alcohol, acetone, amyl alcohol, and acetic ether and insoluble in benzene, chloroform, and ether. Urochrome is said to be a nitrogenous body (4.2 per cent nitrogen), free from iron.

##### B. UROBILIN.

Urobilin, which was at one time considered to be the principal pigment of urine, in reality contributes little toward the pigmentation of this fluid. It is claimed that no urobilin is present in freshly voided normal urine but that its precursor, a chromogen called *urobilinogen*, is present and gives rise to urobilin upon decomposition through the influence of light. It is claimed by some investigators that there are various forms of urobilin, *e. g.*, normal, febrile, physiological, and pathological. Urobilin is said to be very similar to, if not absolutely identical with, hydrobilirubin (see page 179).

Urobilin may be obtained as an amorphous powder which varies in color from brown to reddish-brown, red and reddish-yellow, depend-



ing upon the way in which it is prepared. It is easily soluble in ethyl alcohol, amyl alcohol, and chloroform, and slightly soluble in ether, acetic ether, and in water. Its solutions show characteristic absorption-bands (see Absorption Spectra, Plate II). Under normal conditions urobilin is derived from the bile pigments in the intestine.

Urobilin is increased in most acute infectious diseases such as *erysipelas*, *malaria*, *pneumonia*, and *scarlet fever*. It is also increased in *appendicitis*, *carcinoma of the liver*, *catarrhal icterus*, *pernicious anemia*, and in cases of poisoning by antifebrin, antipyrin, pyridin, and potassium chlorate. In general it is usually increased when blood destruction is excessive and in many disturbances of the liver. It is markedly decreased in phosphorus poisoning.

#### EXPERIMENTS.

1. **Spectroscopic Examination.**—Acidify the urine with hydrochloric acid and allow it to remain exposed to the air for a few moments. By this means if any urobilinogen is present it will be transformed into urobilin. The urine may now be examined by means of the spectroscope. If urobilin is present in the fluid the characteristic absorption-band lying between *b* and *F* will be observed (see Absorption Spectra, Plate II). It may be found necessary to dilute the urine with water before a distinct absorption-band is observed. This test may be modified by acidifying 10 c.c. of urine with hydrochloric acid and shaking it gently with 5 c.c. of amyl alcohol. The alcoholic extract when examined spectroscopically will show the characteristic urobilin absorption-band. (Note the spectroscopic examination in the next experiment.)

2. **Ammoniacal-zinc Chloride Test.**—Render some of the urine ammoniacal by the addition of ammonium hydroxide, and after allowing it to stand a short time filter off the precipitate of phosphates and add a few drops of zinc chloride solution to the filtrate. Observe the production of a greenish fluorescence. Examine the fluid by means of the spectroscope and note the absorption-band which occupies much the same position as the absorption-band of urobilin in acid solution (see Absorption Spectra, Plate II).

3. **Gerhardt's Test.**—To 20 c.c. of urine add 3-5 c.c. of chloroform and shake well. Separate the chloroform extract and add to it a few drops of iodine solution (I in KI). Render the mixture alkaline with dilute solution of potassium hydroxide and note the production of a yellow or yellowish-brown color. The solution ordinarily exhibits a greenish fluorescence.

4. **Wirsing's Test.**—To 20 c.c. of urine add 3-5 c.c. of chloro-

form and shake gently. Separate the chloroform extract and add to it a drop of an alcoholic solution of zinc chloride. Note the rose-red color and the greenish fluorescence. If the solution is turbid it may be rendered clear by the addition of a few c.c. of absolute alcohol.

5. **Ether-Absolute Alcohol Test.**—Mix urine and pure ether in equal volumes and shake gently in a separatory funnel. Separate the ether extract, evaporate it to dryness, and dissolve the residue in 2–3 c.c. of absolute alcohol. Note the greenish fluorescence. Examine the solution spectroscopically and observe the characteristic absorption-band (see Absorption Spectra, Plate II).

6. **Ring Test.**—Acidify 25 c.c. of urine with 2–3 drops of concentrated hydrochloric acid, add 5 c.c. of chloroform and shake the mixture. Separate the chloroform, place it in a test-tube, and add carefully 3–5 c.c. of an alcoholic solution of zinc acetate. Observe the formation of a green ring at the zone of contact of the two fluids. If the tube is shaken a fluorescence may be observed.

#### C. UROERYTHRIN.

This pigment is frequently present in small amount in normal urine. The red color of urinary sediments is due in great part to the presence of uroerythrin. It is easily soluble in amyl alcohol, slightly soluble in acetic ether, absolute alcohol, or chloroform, and nearly insoluble in water. Dilute solutions of uroerythrin are pink in color while concentrated solutions are orange-red or bright red: none of its solutions fluoresce. Uroerythrin is increased in amount after strenuous physical exercise, digestive disturbances, fevers, certain liver disorders, and in various other pathological conditions.

#### PTOMAINES AND LEUCOMAINES.

These toxic substances are said to be present in small amount in normal urine. Very little is known, definitely, however, about them. It is claimed that five different poisons may be detected in the urine, and it is further stated that each of these substances produces a specific and definite symptom when injected intravenously into a rabbit. The resulting symptoms are narcosis, salivation, mydriasis, paralysis, and convulsions. The day urine is principally narcotic and is 2–4 times as toxic as the night urine which is chiefly productive of convulsions.

#### PURINE BASES.

The purine bases found in human urine are adenine, carnine, epiguanine, episarkine, guanine, xanthine, heteroxanthine, hypoxanthine, paraxanthine, and 1-methylxanthine. The main bulk of the purine

base content of the urine is made up of *paraxanthine*, *heteroxanthine* and *1-methylxanthine* which are derived for the most part from the caffeine, theobromine, and theophylline of the food. The total purine base content is made up of the products of two distinct forms of metabolism, *i. e.*, metabolism of ingested nucleins and purines and metabolism of tissue nuclein material. Purine bases resulting from the first form of metabolism are said to be of *exogenous* origin whereas those resulting from the second form of metabolism are said to be of *endogenous* origin. The daily output of purine bases by the urine is extremely small and varies greatly with the individual (16–60 milligrams). The output is increased after the ingestion of nuclein material as well as after the increased destruction of leucocytes. A well marked increase accompanies leukæmia. Edsall has shown that the output of purine bases by the urine is increased as a result of X-ray treatment.

#### EXPERIMENT.

1. **Formation of the Silver Salts.**—Add an excess of magnesia mixture<sup>1</sup> to 25 c.c. of urine. Filter off the precipitate and add ammoniacal silver solution<sup>2</sup> to the filtrate. A precipitate composed of the silver salts of the various purine bases is produced. The purine bases may be determined quantitatively by means of Krüger and Schmidt's method (see p. 429), or Welker's method (see p. 328).

### 2. Inorganic Physiological Constituents.

#### Ammonia.

Next to urea, ammonia is the most important of the nitrogenous end-products of protein metabolism. Ordinarily about 2.5–4.5 per cent of the total nitrogen of the urine is eliminated as ammonia and on the average this would be about 0.7 gram per day. Under normal conditions the ammonia is present in the urine in the form of the *chloride*, *phosphate*, or *sulphate*. This is due to the fact that combinations of this sort are not oxidized in the organism to form urea, but are excreted as such. This explains the increase in the output of ammonia which follows the administration of the ammonium salts of the mineral acids or of the acids themselves. On the other hand, when ammonium acetate and many other ammonium salts of certain organic acids are administered no increase in the output of ammonia occurs since the salt is oxidized and its nitrogen ultimately appears in the urine as urea.

<sup>1</sup> Magnesia mixture may be prepared as follows: Dissolve 175 grams of  $MgSO_4$  and 350 grams of  $NH_4Cl$  in 1400 c.c. of distilled water. Add 700 grams of concentrated  $NH_4OH$ , mix very thoroughly and preserve the mixture in a glass-stoppered bottle.

<sup>2</sup> Ammoniacal silver solution may be prepared according to directions given on page 430.

Recent experiments<sup>1</sup> indicate that the nitrogen in food protein may in part be replaced by ammonium salts.

Copious water drinking increases the ammonia output. This fact has been interpreted as indicating a stimulation of the gastric secretion.<sup>2</sup>

The acids formed during the process of protein destruction within the body have an influence upon the excretion of ammonia similar to that exerted by acids which have been administered. Therefore a pathological increase in the output of ammonia is observed in such diseases as are accompanied by an increased and imperfect protein metabolism, and especially in diabetes, in which disease diacetic acid and  $\beta$ -oxybutyric acid are found in the urine in combination with the ammonia.

As the result of recent experiments Folin claims that a pronounced decrease in the extent of protein metabolism, as measured by the total nitrogen in the urine, is frequently accompanied by a decreased elimination of ammonia. The ammonia elimination is therefore probably determined by other factors than the total protein catabolism as such. Furthermore, he believes that a decided decrease in the total nitrogen excretion is always accompanied by a *relative increase* in the ammonia-nitrogen, provided the food is of a character yielding an alkaline ash.

The quantitative determination of ammonia must be made upon the fresh urine since upon standing the normal urine will undergo ammoniacal fermentation (see page 276).

### Sulphates.

Sulphur in combination is excreted in two forms in the urine; first, as *loosely combined, unoxidized or neutral sulphur*, and, second, as *oxidized or acid sulphur*. The *loosely combined* sulphur is excreted mainly as a constituent of such bodies as cystine, cysteine, taurine, hydrogen sulphide, ethyl sulphide, thiocyanates, sulphonic acids, oxyproteic acid, alloxypoteic acid, and uroferric acid. The amount of loosely combined sulphur eliminated is in great measure independent of the extent of protein decomposition or of the total sulphur excretion. In this characteristic it is somewhat similar to the excretion of creatinine. The *oxidized* sulphur is eliminated in the form of sulphuric acid, principally as salts of sodium, potassium, calcium, and magnesium; a relatively small amount occurs in the form of *etheral* sulphuric acid. *i. e.*, sulphuric acid in combination with such *aromatic* bodies as phenol, indole, skatole, cresol, pyrocatechin, and hydroquinone. Sulphuric acid in combination

<sup>1</sup> Grafe and Schläpfer: *Zeit. physiol. chem.*, 77, 1, 1912, experiments by Abderhalden in same journal.

<sup>2</sup> Wills and Hawk: *Jour. Biol. Chem.*, 9, xxx, 1911 (Proceedings).

with Na, K, Ca or Mg is sometimes termed *inorganic* or *preformed sulphuric acid*, whereas the ethereal sulphuric acid is sometimes called *conjugate sulphuric acid*. The greater part of the sulphur is eliminated in the oxidized form, but the absolute percentage of sulphur excreted as the preformed, ethereal or loosely combined type depends upon the total quantity of sulphur present, *i. e.*, there is no definite ratio between the three forms of sulphur which will apply under all conditions. The preformed sulphuric acid may be precipitated directly from acidified urine with  $\text{BaCl}_2$ , whereas the ethereal sulphuric acid must undergo a preliminary boiling in the presence of a mineral acid before it can be so precipitated.

The sulphuric acid excreted in the urine arises principally from the oxidation of protein material within the body; a relatively small amount is due to ingested sulphates. Under normal conditions about 2.5 grams of sulphuric acid is eliminated daily. Since the sulphuric acid content of the urine has, for the most part, a protein origin and since one of the most important constituents of the protein molecule is nitrogen, it would be reasonable to suppose that a fairly definite ratio might exist between the excretion of these two elements. However, when we appreciate that the percentage content of N and S present in different proteins is subject to rather wide variations, the fixing of a ratio which will express the exact relation existing between these two substances, as they appear in the urine as end-products of protein metabolism, is practically impossible. It has been suggested that the ratio 5:1 expresses this relation in a general way.

Pathologically, the excretion of sulphuric acid by the urine is increased in acute fevers and in all other diseases marked by a stimulated metabolism, whereas a decrease in the sulphuric acid excretion is observed in those diseases which are accompanied by a loss of appetite and a diminished metabolic activity.

#### EXPERIMENTS.

1. **Detection of Inorganic Sulphuric Acid.**—Place about 10 c.c. of urine in a test-tube, acidify with acetic acid and add some barium chloride solution. A white precipitate of barium sulphate forms.

2. **Detection of Ethereal Sulphuric Acid.**—Filter off the barium sulphate precipitate formed in the above experiment, add 1 c.c. of hydrochloric acid and a little barium chloride solution to the filtrate and heat the mixture to boiling for 1–2 minutes. Note the appearance of a turbidity due to the presence of sulphuric acid which has been separated from the ethereal sulphates and has combined with the barium of the  $\text{BaCl}_2$  to form  $\text{BaSO}_4$ .

3. **Detection of Loosely Combined or Neutral Sulphur.**—Place about 10 c.c. of urine in a test-tube, introduce a small piece of zinc, add sufficient hydrochloric acid to cause a gentle evolution of hydrogen, and over the mouth of the tube place a filter paper saturated with lead acetate solution. In a short time the portion of the paper in contact

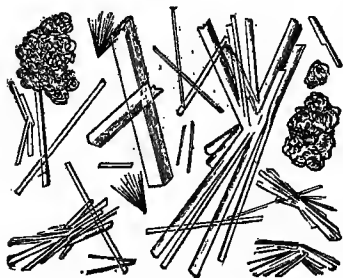


FIG. 100.—CALCIUM SULPHATE.  
(Hensel and Weil.)

with the vapors within the test-tube becomes blackened due to the formation of lead sulphide. The nascent hydrogen has reacted with the loosely combined or neutral sulphur to form hydrogen sulphide and this gas coming in contact with the lead acetate paper has caused the production of the black lead sulphide. Sulphur in the form of inorganic or ethereal sulphuric acid does not respond to this test.

4. **Calcium Sulphate Crystals.**—Place 10 c.c. of urine in a test-tube, add 10 drops of calcium chloride solution and allow the tube to stand until crystals form. Examine the calcium sulphate crystals under the microscope and compare them with those shown in Fig. 100, above.

## Chlorides.

Next to urea, the chlorides constitute the chief *solid* constituent of the urine. The principal chlorides found in the urine are those of sodium, potassium, ammonium, and magnesium, with sodium chloride predominating. The excretion of chloride is dependent, in great part, upon the nature of the diet, but on the average the daily output is about 10–15 grams, expressed as sodium chloride. Copious water-drinking increases the output of chlorides considerably. Because of their solubility, chlorides are never found in the urinary sediment.

Since the amount of chlorides excreted in the urine is due primarily to the chloride content of the food ingested, it follows that a decrease in the amount of ingested chloride will likewise cause a decrease in the chloride content of the urine. In cases of actual fasting the chloride content of the urine may be decreased to a *slight trace* which is derived from the body fluids and tissues. Under these conditions, however, an examination of the blood of the fasting subject will show the percentage of chlorides in this fluid to be approximately normal. This forms a very striking example of the care nature takes to maintain the normal composition of the blood. There is a limit to the power of the

body to maintain this equilibrium, however, and if the fasting organism be subjected to the influence of diuretics for a time, a point is reached where the composition of the blood can no longer be maintained and a gradual decrease in its chloride content occurs which finally results in death. Death is supposed to result not so much because of a lack of chlorine as from a *deficiency of sodium*. This is shown from the fact that potassium chloride, for instance, cannot replace the sodium chloride of the blood when the latter is decreased in the manner above stated. When this substitution is attempted the potassium salt is excreted at once in the urine, and death follows as above indicated.

Pathologically, the excretion of chlorides may be decreased in some fevers, chronic nephritis, croupous pneumonia, diarrhoea, certain stomach disorders, and in acute articular rheumatism.

#### EXPERIMENT.

**Detection of Chlorides in Urine.**—Place about 5 c.c. of urine in a test-tube, render it acid with nitric acid and add a few drops of a solution of silver nitrate. A white precipitate, due to the formation of silver chloride, is produced. This precipitate is soluble in ammonium hydroxide.

#### Phosphates.

Phosphoric acid exists in the urine in two general forms: First, that in combination with the alkali metals, sodium and potassium, and the radical ammonium; second, that in combination with the alkaline earths, calcium and magnesium. Phosphates formed through a union of phosphoric acid with the alkali metals are termed *alkaline phosphates*, or phosphates of the alkali metals, whereas phosphates formed through a union of phosphoric acid with the alkaline earths are termed *earthy phosphates*, or phosphates of the alkaline earths.

Three series of salts are formed by phosphoric acid: *Normal*,  $M_3PO_4$ ,<sup>1</sup> *mono-hydrogen*,  $M_2HPO_4$ , and *di-hydrogen*,  $MH_2PO_4$ . The di-hydrogen salts are acid in reaction, and it was generally believed that about 60 per cent of the total phosphate content of the urine was in the form of this type of salt, and that the acidity of the urine was due in great part to the presence of *sodium di-hydrogen phosphate*. Recently, however, it has been quite clearly shown that the normal acidity of the urine is not due to the presence of this salt, but is due, at least in part, to the presence of various acidic radicals. In this connection Folin believes that the phosphates in clear acid urine are *all* of the *mono-hydrogen* type, and that the acidity of the urines of this character is generally greater than the

<sup>1</sup> M may be occupied by any of the alkali metals or alkaline earths.

combined acidity of all the phosphates present; the excess in the acidity above that due to phosphates be believed to be due to *free organic acids*. Henderson<sup>1</sup> maintains that "determinations of hydrogen ionization in urine and its behavior toward indicators both support the view that in urine there exists a mixture of *mono-* and *di-hydrogen* phosphates of sodium, ammonium and other bases." The observation has recently been made that urine may be separated into two portions, one part consisting almost entirely of inorganic matter including practically *all of the phosphates* and having an *alkaline reaction*, the other containing practically all of the *organic substances* and no phosphates and having an *acid reaction*.

In bones the phosphates occur principally in the form of the normal salts of calcium and magnesium. The mono-hydrogen salts as a class are alkaline in reaction to litmus, and it is to the presence of di-sodium hydrogen phosphate,  $\text{Na}_2\text{HPO}_4$ , that the greater part of the alkalinity of the saliva is due.

The excretion of phosphoric acid is extremely variable, but on the average the total output for 24 hours is about 2.5 grams, expressed as  $\text{P}_2\text{O}_5$ . Ordinarily the total output is distributed between alkaline phosphates and earthy phosphates approximately in the ratio 2:1. The greater part of this phosphoric acid arises from the ingested food, either from the preformed phosphates or more especially from the phosphorus in organic combination such as we find it in *phospho-proteins*, *nucleo-proteins* and *lecithins*; the phosphorus-containing tissues of the body also contribute to the total output of this element. Alkaline phosphates ingested with the food have a tendency to increase the phosphoric acid content of the urine to a greater extent than the earthy phosphates so ingested. This is due, in a measure, to the fact that a portion of the earthy phosphates, under certain conditions, may be precipitated in the intestine and excreted in the feces; this is especially to be noted in the case of herbivorous animals. Since the extent to which the phosphates are absorbed in the intestine depends upon the form in which they are present in the food, under ordinary conditions, there can be no absolute relationship between the urinary output of nitrogen and phosphorus. If the diet is constant, however, from day to day, thus allowing of the preparation of both a nitrogen and a phosphorus balance,<sup>2</sup> a definite ratio may be established. In experiments upon dogs, which were fed an exclusive meat diet, the ratio of nitrogen to phosphorus, in the urine and feces, was found to be 8.1:1.

<sup>1</sup> Henderson: *Am. Jour. Physiol.*, 15, 257, 1906.

<sup>2</sup> In metabolism experiments, a statement showing the relation existing between the nitrogen content of the food on the one hand and that of the urine and feces on the other, for a definite period, is termed a *nitrogen balance* or a "balance of the income and outgo of nitrogen."



It has been demonstrated by recent investigation that the ingestion of *inorganic* phosphorus compounds may give rise to *organic* phosphorus compounds such as lecithin, phosphatides, nucleoproteins and phosphoproteins. This is an instance of an organic substance synthesized from an inorganic substance. The experiments have been made principally on ducks<sup>1</sup> and hens.<sup>2</sup>

Pathologically the excretion of phosphoric acid is increased in such diseases of the bones as diffuse periostosis, osteomalacia, and rickets; according to some investigators, in the early stages of pulmonary tuberculosis, in acute yellow atrophy of the liver, in diseases which are accompanied by an extensive decomposition of nervous tissue, and after sleep induced by potassium bromide or chloral hydrate (Mendel). It is also increased after copious water-drinking. A decrease in the excretion of phosphates is at times noted in febrile affections, such as the acute infectious diseases; in pregnancy, in the period during which the foetal bones are forming, and in diseases of the kidneys, because of non-elimination.

#### EXPERIMENTS.

1. **Formation of "Triple Phosphate."**—Place some urine in a beaker, render it alkaline with ammonium hydroxide, add a small



FIG. 101.—"TRIPLE PHOSPHATE." (Ogden.)

amount of magnesium sulphate solution and allow the beaker to stand in a cool place over night. Crystals of *ammonium magnesium phosphate*, "triple phosphate," form under these conditions. Examine the crystalline sediment under the microscope and compare the forms of the crystals with those shown in Fig. 101, above.

2. **"Triple Phosphate" Crystals in Ammoniacal Fermentation.**—Stand some urine aside in a beaker for several days. Ammoniacal

<sup>1</sup> Fingerling: *Biochem. Zeit.*, 38, 448, 1912.

<sup>2</sup> McCollum and Halpin: *Jour. Biol. Chem.*, 11, 47 (Proceedings), 1912.

fermentation will develop and "triple phosphate" crystals will form. Examine the sediment under the microscope and compare the crystals with those shown in Fig. 101, below.

3. **Detection of Earthy Phosphates.**—Place 10 c.c. of urine in a test-tube and render it alkaline with ammonium hydroxide. Warm the mixture and note the separation of a precipitate of *earthy phosphates*.

4. **Detection of Alkaline Phosphates.**—Filter off the earthy phosphates as formed in the last experiment, and add a small amount of magnesia mixture (see page 313) to the filtrate. Now warm the mixture and observe the formation of a white precipitate due to the presence of alkaline phosphates. Note the difference in the size of the precipitates of the two forms of phosphates from this same volume of urine. Which form of phosphates was present in the larger amount, *earthy* or *alkaline*?

5. **Influence upon Fehling's Solution.**—Place 2 c.c. of Fehling's solution in a test-tube, dilute it with 4 volumes of water and heat to boiling. Add a solution of sodium dihydrogen phosphate,  $\text{NaH}_2\text{PO}_4$ , a small amount at a time, and heat after each addition. What do you observe? What does this observation force you to conclude regarding the interference of phosphates in the testing of *diabetic* urine by means of Fehling's test?

### Sodium and Potassium.

The elements sodium and potassium are always present in the urine. Usually they are combined with such acidic radicals as  $\text{Cl}$ ,  $\text{CO}_3$ ,  $\text{SO}_4$ , and  $\text{PO}_4$ . The amount of potassium, expressed as  $\text{K}_2\text{O}$ , excreted in 24 hours by an adult, subsisting upon a mixed diet, is on the average 2–3 grams, whereas the amount of sodium, expressed as  $\text{Na}_2\text{O}$ , under the same conditions, is ordinarily 4–6 grams. The ratio of K to Na is generally about 3 : 5. The absolute quantity of these elements excreted depends, of course, in large measure, upon the nature of the diet. Because of the non-ingestion of  $\text{NaCl}$  and the accompanying destruction of potassium-containing body tissues, the urine during fasting contains more potassium salts than sodium salts.

Pathologically the output of potassium, in its relation to sodium, may be increased during fever; following the crisis, however, the output of this element may be decreased. It may also be increased in conditions associated with *acid intoxication*.

### Calcium and Magnesium.

The greater part of the calcium and magnesium excreted in the urine is in the form of phosphates. The daily output, which depends

principally upon the nature of the diet, aggregates on the average about 1 gram and is made up of the phosphates of calcium and magnesium in the proportion of 1:2. The percentage of calcium salts present in the urine at any one time forms no dependable index as to the absorption of this class of salts, since they are again excreted into the intestine after absorption. It is therefore impossible to draw any satisfactory conclusions regarding the excretion of the alkaline earths unless we obtain accurate analytical data from both the feces and the urine.

Very little is known positively regarding the actual course of the excretion of the alkaline earths under pathological conditions except that an excess of calcium is found in *acid intoxication* and some diseases of the bones.

### Carbonates.

Carbonates generally occur in small amount in the urine of man and carnivora under normal conditions, whereas much larger quantities are ordinarily present in the urine of herbivora. The alkaline reaction of the urine of herbivora is dependable in great measure upon the presence of carbonates. In general a urine containing carbonates in appreciable amount is turbid when passed or becomes so shortly after. These bodies ordinarily occur as alkali or alkaline earth compounds and the turbid character of urine containing them is usually due principally to the latter class of substances. The carbonates of the alkaline earths are often found in amorphous urinary sediments.

### Iron.

Iron is present in small amount in normal urine. It probably occurs partly in inorganic and partly in organic combination. The iron contained in urinary pigments or chromogens is in organic combination. According to different investigators the iron content of normal urine will probably not average more than 0.001 gram per day.

### EXPERIMENT.

**Detection of Iron in Urine.**—Evaporate a convenient volume (10-15 c.c.) of urine to dryness. Incinerate and dissolve the residue in a few drops of iron-free hydrochloric acid and dilute the acid solution with 5 c.c. of water. Divide the acid solution into two parts and make the following tests: (a) To the first part add a solution of ammonium thiocyanate; a red color indicates the presence of iron. (b) To the second part of the solution add a little potassium ferrocyanide solution; a precipitate of Prussian blue forms upon standing.

### Fluorides, Nitrates, Silicates and Hydrogen Peroxide.

These substances are all found in traces in human urine under normal conditions. Nitrates are undoubtedly introduced into the organism in the water and ingested food. The average excretion of nitrates is about 0.5 gram per day, the output being the largest upon a vegetable diet and smallest upon a meat diet. Nitrites are found only in urine which is undergoing decomposition and are formed from nitrates in the course of ammoniacal fermentation. Hydrogen peroxide has been detected in the urine, but its presence is believed to possess no pathological importance.

## CHAPTER XIX.

### URINE: PATHOLOGICAL CONSTITUENTS.<sup>1</sup>

Dextrose.			
	{	Serum albumin.	
		Serum globulin.	
Proteins	{	Proteoses	
		{	Deutero-proteose.
			Hetero-proteose.
		"Bence-Jones' protein."	
		Peptone.	
	Nucleoprotein.		
	Fibrin.		
	Oxyhæmoglobin.		
Blood	{	Form elements.	
		Pigment.	
Bile	{	Pigments.	
		Acids.	
Creatine. <sup>2</sup>			
Acetone.			
Diacetic acid.			
$\beta$ -Oxybutyric acid.			
Conjugate glycuronates.			
Pentoses.			
Fat.			
Hæmatoporphyrin.			
Lactose.			
Galactose.			
Lævulose.			
Inosite.			
Laiose.			
Melanin.			
Urorosein.			
Unknown substances.			

### DEXTROSE.

Traces of this sugar occur in normal urine, but the amount is not sufficient to be readily detected by the ordinary simple qualitative

<sup>1</sup> See note at the bottom of page 283.

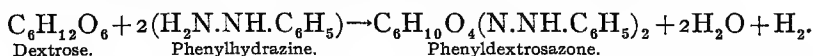
<sup>2</sup> Normal constituent of urine of infants and children.

tests. There are two distinct types of *pathological* glycosuria, *i. e.*, transitory glycosuria and persistent glycosuria. The transitory type may follow the ingestion of an excess of sugar, causing the *assimilation limit*<sup>1</sup> to be exceeded, or it may accompany any one of several disorders which cause impairment of the power of assimilating sugar. In the persistent type large amounts of sugar are excreted daily in the urine for long periods of time. Under such circumstances a condition known as diabetes mellitus exists. Ordinarily, diabetic urine which contains a high percentage of sugar possesses a faint yellow color, a high specific gravity, and a volume which is above normal.

#### EXPERIMENTS.

1. **Phenylhydrazine Reaction.**—Test the urine according to one of the following methods: (a) To a small amount of phenylhydrazine mixture, furnished by the instructor,<sup>2</sup> add 5 c.c. of the urine, shake well, and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the tube to cool *slowly* and examine the crystals microscopically (Plate III, opposite page 28). If the solution has become too concentrated in the boiling process it will be light-red in color and no crystals will separate until it is diluted with water.

Yellow crystalline bodies called *osazones* are formed from certain sugars under these conditions, in general each individual sugar giving rise to an osazone of a definite crystalline form which is typical for that sugar. It is important to remember in this connection that, of the simple sugars of interest in physiological chemistry, dextrose and lævulose yield the same osazone, with phenylhydrazine. Each osazone has a definite melting-point, and as a further and more accurate means of identification it may be recrystallized and identified by the determination of its melting-point and nitrogen content. The reaction taking place in the formation of phenyldextrosazone is as follows:



(b) Place 5 c.c. of the urine in a test-tube, add 1 c.c. of phenylhydrazine-acetate solution furnished by the instructor,<sup>3</sup> and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the liquid to cool *slowly* and examine the crystals microscopically (Plate III, opposite p. 28).

<sup>1</sup>The assimilation limit for dextrose has been shown to be between 100 and 150 grams (Brasch: *Zeit. für Biol.*, 50, 113, 1907).

<sup>2</sup>This mixture is prepared by combining one part of phenylhydrazine-hydrochloride and two parts of sodium acetate, by *weight*. These are thoroughly mixed in a mortar.

<sup>3</sup>This solution is prepared by mixing one part *by volume*, in each case, of glacial acetic acid, one part of water and two parts of phenylhydrazine (the base):

The phenylhydrazine test has been so modified by Cipollina as to be of use as a *rapid clinical test*. The directions for this test are given in the next experiment.

2. **Cipollina's Test.**—Thoroughly mix 4 c.c. of urine, 5 drops of phenylhydrazine (the base) and  $\frac{1}{2}$  c.c. of glacial acetic acid in a test-tube. Heat the mixture for about one minute over a low flame, shaking the tube continually to prevent loss of fluid by bumping. Add 4–5 drops of potassium hydroxide or sodium hydroxide (sp. gr. 1.16), being certain that the fluid in the test-tube remains acid; heat the mixture again for a moment and then cool the contents of the tube. Ordinarily the crystals form at once, especially if the urine possesses a low specific gravity. If they do not appear immediately allow the tube to stand at least 20 minutes before deciding upon the absence of sugar.

Examine the crystals under the microscope and compare them with those shown in Plate III, opposite page 28.

3. **Riegler's Reaction.**<sup>1</sup>—Introduce 0.1 gram of phenylhydrazine-hydrochloride and 0.25 gram of sodium acetate into a test-tube, add 20 drops of the urine under examination, and heat the mixture to boiling. Now introduce 10 c.c. of a 3 per cent solution of potassium hydroxide and gently shake the tube and contents. If the urine under examination contains dextrose the liquid in the tube will assume a red color. One per cent dextrose yields an immediate color whereas 0.05 per cent yields the color only after the lapse of a period of one-half hour from the time the alkali is added. If the color appears after the 30-minute interval the color change is without significance inasmuch as sugar-free urines will respond thus. The reaction is given by all aldehydes and therefore the test cannot be safely employed in testing urines preserved by formaldehyde. Albumin does not interfere with the test.

4. **Bottu's Test.**<sup>2</sup>—To 8 c.c. of Bottu's reagent<sup>3</sup> in a test-tube add 1 c.c. of the urine under examination and mix the liquids by gentle shaking. Now heat the upper portion of the mixture to boiling, add an additional 1 c.c. of urine and heat the mixture again immediately. The appearance of a blue color accompanied by the precipitation of small particles of indigo blue indicates the presence of dextrose in the urine under examination. The test will serve to detect the presence of 0.1 per cent of dextrose and is uninfluenced by creatinine or by ammonium salts.

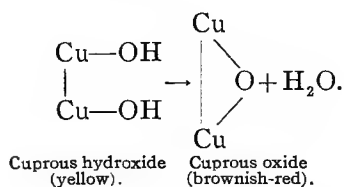
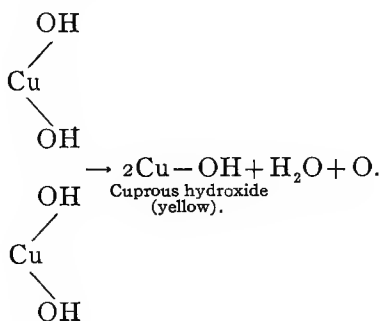
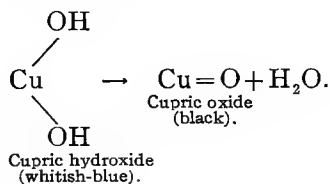
5. **Reduction Tests.**—To their aldehyde or ketone structure many sugars owe the property of readily reducing the alkaline solutions of the

<sup>1</sup> Riegler: *Compt. rend. soc. biol.*, 66, p. 795.

<sup>2</sup> Bottu: *Compt. rend. soc. biol.*, 66, p. 972.

<sup>3</sup> This reagent contains 3.5 grams of *o*-nitrophenylpropionic acid and 5 c.c. of a freshly prepared 10 per cent solution of sodium hydroxide per liter.

oxides of metals like copper, bismuth, and mercury; they also possess the property of reducing ammoniacal silver solutions with the separation of metallic silver. Upon this property of reduction the most widely used tests for sugars are based. When whitish-blue cupric hydroxide in suspension in an alkaline liquid is heated it is converted into insoluble black cupric oxide, but if a reducing agent like certain sugars be present the cupric hydroxide is reduced to insoluble yellow cuprous hydroxide, which in turn on further heating may be converted into brownish-red or red cuprous oxide. These changes are indicated as follows:



The chemical equations here discussed are exemplified in Trommer's and Fehling's tests.

(a) *Trommer's Test*.—To 5 c.c. of urine in a test-tube add one-half its volume of KOH or NaOH. Mix thoroughly and add, drop by drop, agitating after the addition of each drop, a *very dilute* solution of copper sulphate. Continue the addition until there is a slight permanent precipitate of cupric hydroxide and in consequence the solution is slightly turbid. Heat, and the cupric hydroxide is reduced to yellow cuprous hydroxide or to brownish-red cuprous oxide. If the solution



of copper sulphate used is too strong, a small brownish-red precipitate produced in the presence of a low percentage of dextrose may be entirely masked. On the other hand, if too little copper sulphate is used a light-colored precipitate formed by uric acid and purine bases may obscure the brownish-red precipitate of cuprous oxide. The action of KOH or NaOH in the presence of an excess of sugar and insufficient copper will produce a brownish color. Phosphates of the alkaline earths may also be precipitated in the alkaline solution and be mistaken for cuprous hydroxide. Trommer's test is not very satisfactory.

Salkowski<sup>1</sup> has very recently proposed a modification of the Trommer procedure which he claims is a very accurate sugar test.

(b) *Fehling's Test*.—To about 1 c.c. of Fehling's solution<sup>2</sup> in a test-tube add about 4 c.c. of water, and boil. This is done to determine whether the solution will of itself cause the formation of a precipitate of brownish-red cuprous oxide. If such a precipitate forms, the Fehling's solution must not be used. Add urine to the warm Fehling's solution, *a few drops* at a time, and heat the mixture after each addition. The production of yellow cuprous hydroxide or brownish-red cuprous oxide indicates that reduction has taken place. The yellow precipitate is more likely to occur if the urine is added rapidly and in large amount, whereas with a less rapid addition of smaller amounts of urine the brownish-red precipitate is generally formed.

This is a much more satisfactory test than Trommer's, but even this test is not entirely reliable when used to detect sugar in the urine. Such bodies as *conjugate glycuronates, uric acid, nucleoprotein, and homogentisic acid*, when present in sufficient amount, may produce a result similar to that produced by sugar. *Phosphates of the alkaline earths* may be precipitated by the alkali of the Fehling's solution and in appearance may be mistaken for the cuprous hydroxide. Cupric hydroxide may also be reduced to cuprous oxide and this in turn be dissolved by *creatinine*, a normal urinary constituent. This will give the urine under examination a greenish tinge and may obscure the sugar reaction even when a considerable amount of sugar is present. According to Laird<sup>3</sup> even small amounts of creatinine will *retard the reaction velocity* of reducing sugars with Fehling's solution.

<sup>1</sup> Salkowski: *Zeit. physiol. Chem.*, 79, 164, 1912.

<sup>2</sup> Fehling's solution is composed of two definite solutions—a copper sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

*Copper sulphate solution* = 34.65 grams of copper sulphate dissolved in water and made up to 500 c.c.

*Alkaline tartrate solution* = 125 grams of potassium hydroxide and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

<sup>3</sup> Laird: *Journ. Path. and Bact.*, 16, 398, 1912.

(c) *Benedict's Modifications of Fehling's Test. First Modification*  
 To 2 c.c. of Benedict's solution<sup>1</sup> in a test-tube add 6 c.c. of dist water and 7-9 drops (not more) of the urine under examination. Shake the mixture vigorously for about 15-30 seconds and permit it to cool to room temperature spontaneously. (If desired this process may be repeated, although it is ordinarily unnecessary.) If sugar is present in the solution a precipitate will form which is often *bluish-green* at first, especially if the percentage of sugar is low, and which usually becomes *yellowish* upon standing. If the sugar present exceeds 0.06 per cent this precipitate generally forms at or below the boiling point, whereas if less than 0.06 per cent of sugar is present the precipitate forms more slowly and generally only after the solution has cooled. The greenish precipitate obtained with urines containing small amounts of sugar may be a compound of copper with the sugar or a compound of some constituent of the urine with reduced copper oxide instead of being a precipitate of cuprous hydroxide or oxide as is the case with the original Fehling solution is reduced.

Benedict claims that, whereas the original Fehling's test will serve to detect sugar when present in a concentration of less than 0.1 per cent, that the above modification will serve to detect sugar when present in as small quantity as 0.015-0.02 per cent. This claim has been corroborated recently by Harrison.<sup>2</sup> The modified solution used in the above test differs from the original in that 100 grams of sodium borate is substituted for the 125 grams of potassium hydroxide commonly used, thus forming a Fehling solution which is considerably *alkaline* than the original. This alteration in the composition of the Fehling solution is of advantage in the detection of sugar in the urine inasmuch as the strong alkalinity of the ordinary Fehling solution has a tendency, when the reagent is boiled with a urine containing a small amount of dextrose, to decompose sufficient of the sugar to render the detection of the remaining portion exceedingly difficult by the usual technique. Benedict claims that for this reason the use of his modified solution permits the detection of smaller amounts of sugar than does the use of the ordinary Fehling solution. Benedict has further modified his solution for use in the quantitative determination of sugar (see page 385).

<sup>1</sup> Benedict's modified Fehling solution consists of two definite solutions—a copper sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

*Copper sulphate solution* = 34.65 grams of copper sulphate dissolved in water and made up to 500 c.c.

*Alkaline tartrate solution* = 100 grams of anhydrous sodium carbonate and 173 grams Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and used in equal volumes when needed for use. This is done to prevent deterioration.

<sup>2</sup> Harrison: *Pharm. Jour.*, 87, 746, 1911.

*Second Modification*<sup>1</sup>—Very recently Benedict has further modified his solution and has succeeded in obtaining one which does not deteriorate upon long standing.<sup>2</sup> The following is the procedure for the detection of dextrose in the urine: To 5 c.c. of the reagent in a test-tube add eight (not more) drops of the urine to be examined. The fluid is then boiled vigorously for from one to two minutes and then allowed to cool *spontaneously*. In the presence of dextrose *the entire body of the solution will be filled* with a precipitate, which may be *red, yellow, or green* in color, depending upon the amount of sugar present. If no dextrose is present, the solution will either remain perfectly clear, or will show a very faint turbidity, due to precipitated urates. Even very small quantities of dextrose in urine (0.1 per cent) yield precipitates of surprising bulk with this reagent, and the positive reaction for dextrose is the filling of the entire body of the solution with a precipitate, so that the solution becomes opaque. Since *amount* rather than color of the precipitate is made the basis of this test, it may be applied, even for the detection of small quantities of dextrose, as readily in artificial light as in daylight.

(d) *Allen's Modification of Fehling's Test*.—The following procedure is recommended: "From 7 to 8 c.c. of the sample of urine to be tested is heated to boiling in a test-tube, and, without separating any precipitate of albumin which may be produced, 5 c.c. of the solution of copper sulphate used for preparing Fehling's solution is added. This produces a precipitate containing uric acid, xanthine, hypoxanthine, phosphates, etc. To render the precipitation complete, however, it is desirable to add to the liquid, *when partially cooled*, from 1 to 2 c.c. of a saturated solution of sodium acetate having a feebly acid reaction to litmus.<sup>3</sup> The liquid is filtered and to the filtrate, which will have a bluish-green color, 5 c.c. of the alkaline tartrate mixture used for preparing Fehling's solution is added, and the liquid boiled for 15–20 seconds.

<sup>1</sup> Benedict: *Jour. Am. Med. Ass'n.*, 57, 1193, 1911.

<sup>2</sup> Benedict's new solution has the following composition:

Copper sulphate .....	17.3 gm.
Sodium citrate .....	173.0 gm.
Sodium carbonate (anhydrous) .....	100.0 gm.
Distilled water to .....	1000.0 c.c.

With the aid of heat dissolve the sodium citrate and carbonate in about 600 c.c. of water. Pour (through a folded filter if necessary) into a glass graduate and make up to 850 c.c. Dissolve the copper sulphate in about 100 c.c. of water and make up to 150 c.c. Pour the carbonate-citrate solution into a large beaker or casserole and add the copper sulphate solution slowly, with constant stirring. The mixed solution is ready for use, and does not deteriorate upon long standing.

<sup>3</sup> Sufficient acetic acid should be added to the sodium acetate solution to render it feebly acid to litmus. A saturated solution of sodium acetate keeps well, but weaker solutions are apt to become mouldy, and then possess the power of reducing Fehling's solution. Hence it is essential in all cases of importance to make a blank test by mixing equal measures of copper sulphate solution, alkaline tartrate solution and water, adding a little sodium acetate solution, and heating the mixture to boiling.

In the presence of more than 0.25 per cent of sugar, separation of cuprous oxide occurs before the boiling-point is reached; but with smaller quantities precipitation takes place during the cooling of the solution, which becomes greenish, opaque, and suddenly deposits cuprous oxide as a fine brownish-red precipitate."

(e) *Boettger's Test*.—To 5 c.c. of urine in a test-tube add 1 c.c. of KOH or NaOH and a very small amount of bismuth subnitrate, and boil. The solution will gradually darken and finally assume a black color due to reduced bismuth. If the test is made with urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced (bismuth sulphide).

(f) *Nylander's Test (Almén's Test)*.—To 5 c.c. of urine in a test-tube add one-tenth its volume of Nylander's reagent<sup>1</sup> and heat for five minutes in a boiling water-bath.<sup>2</sup> The mixture will darken if reducing sugar is present and upon standing for a few moments a black color will appear. This color is due to the precipitation of bismuth. If the test is made on urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced. Dextrose when present to the extent of 0.08 per cent may be detected by this reaction. It is claimed by Bechold that Nylander's and Boettger's tests give a negative reaction with solutions containing sugar when mercuric chloride or chloroform is present. Other observers<sup>3</sup> have failed to verify the inhibitory action of the mercuric chloride and have shown that the inhibitory influence of chloroform may be overcome by raising the temperature of the urine to the boiling-point for a period of five minutes previous to making the test.

Urines rich in *indican*, *uroerythrin*, *urochrome* or *hæmatoporphyrin*, as well as urines excreted after the ingestion of large amounts of certain *medicinal substances*, may give a darkening of Nylander's reagent similar to that of a true sugar reaction. It is a disputed point whether the urine after the administration of urotropin will reduce Nylander's reagent.<sup>4</sup>

Strausz<sup>5</sup> has recently shown that the urine of diabetics to whom "Iothion" (diiodohydroxypropane) has been administered will give a negative Nylander's reaction and respond positively to the Fehling and

<sup>1</sup> Nylander's reagent is prepared by digesting 2 grams of bismuth subnitrate and 4 grams of Rochelle salt in 100 c.c. of a 10 per cent potassium hydroxide solution. The reagent is then cooled and filtered.

<sup>2</sup> Hammarsten suggests that the solution be boiled for 2-5 minutes (according to the sugar content) over a free flame and the tube then permitted to stand five minutes before drawing conclusions.

<sup>3</sup> Rehfuss and Hawk: *Jour. Biol. Chem.*, 7, 267, 1910; also Zeidlitz: *Upsala Lakäreforen Forh.*, N. F., 11, 1906.

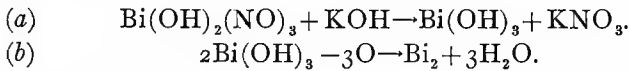
<sup>4</sup> Abt: *Archives of Pediatrics*, 24, 275, 1907; also Weitbrecht: *Schweiz. Woch.*, 47, 577, 1909.

<sup>5</sup> Strausz: *Münch med. Woch.*, 59, 85, 1912.

polarization tests. "Iothion" also interferes with the Nylander test *in vitro* whereas KI and I do not.

According to Rustin and Otto the addition of  $\text{PtCl}_4$  increases the delicacy of Nylander's reaction. They claim that this procedure causes the sugar to be converted *quantitatively*. No quantitative method has yet been devised, however, based upon this principle.

A positive Nylander or Boettger test is probably due to the following reactions:



Bohmansson,<sup>1</sup> before testing the urine under examination treats it (10 c.c.) with 1/5 volume of 25 per cent hydrochloric acid and 1/2 volume of bone black. This mixture is shaken one minute, then filtered, and the neutralized filtrate tested by Nylander's reaction. Bohmansson claims that this procedure removes certain interfering substances, notably urochrome.

6. **Fermentation Test.**—Rub up in a mortar about 15 c.c. of the urine with a small piece of compressed yeast. Transfer the mixture to a saccharometer (Fig. 3, p. 36) and stand it aside in a warm place for about 12 hours. If dextrose is present, alcoholic fermentation will occur and carbon dioxide will collect as a gas in the upper portion of the tube. On the completion of fermentation introduce, by means of a bent pipette, a little KOH solution into the graduated portion, place the thumb tightly over the opening in the apparatus and invert the saccharometer. Explain the result.

The important findings of Neuberg and associates<sup>2</sup> recently reported indicate very clearly that the liberation of carbon dioxide by yeast is not necessarily a criterion of the presence of sugar. The presence of a new enzyme called *carboxylase* has been demonstrated in yeast which has the power of *splitting off CO<sub>2</sub> from the carboxyl group of amino and other aliphatic acids.*

7. **Barfoed's Test.**—Place about 5 c.c. of Barfoed's solution<sup>3</sup> in a test-tube and heat to boiling. Add the urine under examination slowly, a few drops at a time, heating after each addition. Reduction is indicated by the production of a red precipitate. If the precipitate does not form upon continued boiling allow the tube to stand a few minutes and examine. NaCl interferes with this test (Welker).

Barfoed's test is *not* a specific test for dextrose as is frequently stated,

<sup>1</sup> Bohmansson: *Biochem. Zeit.*, 19, p. 281.

<sup>2</sup> Neuberg and Associates: *Biochem. Zeitsch.*, 31, 170; 32, 323; 36, (60, 68, 76), 1911.

<sup>3</sup> Barfoed's solution is prepared as follows: Dissolve 4.5 grams of neutral, crystallized copper acetate in 100 c.c. of water and add 1.2 c.c. of 50 per cent acetic acid.

but simply serves to detect *monosaccharides*. Disaccharides will also respond to the test, according to Hinkel and Sherman,<sup>1</sup> if the solution is boiled sufficiently long in contact with the reagent to *hydrolyze the disaccharide* through the action of the acetic acid present in the Barfoed's solution.

Mathews and McGuigan<sup>2</sup> have also shown that disaccharides will respond to this test under proper conditions of acidity.

8. **Polariscopic Examination.**—For directions as to the use of the polariscope see page 36.

## PROTEINS.

Normal urine contains a trace of protein material but the amount present is so slight as to escape detection by any of the simple tests in general use for the detection of protein urinary constituents. The following are the more important forms of protein material which have been detected in the urine under pathological conditions:

- (1) Serum albumin.
- (2) Serum globulin.
- (3) Proteoses { Deutero-proteose.  
Hetero-proteose.  
"Bence-Jones' protein."
- (4) Peptone.
- (5) Nucleoprotein.
- (6) Fibrin.
- (7) Oxyhæmoglobin.

## ALBUMIN.

Albuminuria is a condition in which serum albumin or serum globulin appears in the urine. There are two distinct forms of albuminuria, *i. e.*, *renal* albuminuria and *accidental* albuminuria. Sometimes the terms "true" albuminuria and "false" albuminuria are substituted for those just given. In the renal type the albumin is excreted by the kidneys. This is the more serious form of the malady and at the same time is more frequently encountered than the accidental type. Among the causes of renal albuminuria are altered blood pressure in the kidneys, altered kidney structure, or changes in the composition of the blood entering the kidneys, thus allowing the albumin to diffuse more readily. In the accidental form of albuminuria the albumin is *not* excreted by the kidneys as is the case in the renal form of the disorder, but arises from the blood,

<sup>1</sup> Hinkel & Sherman: *Journ. Am. Chem. Soc.*, 29, 1744, 1907.

<sup>2</sup> Mathews and McGuigan: *Amer. Journ. Physiol.*, 19, 175, 1907.

lymph, or some albumin-containing exudate coming into contact with the urine at some point below the kidneys.

#### EXPERIMENTS.

**Heller's Ring Test.**—Place 5 c.c. of concentrated  $\text{HNO}_3$  in a test-tube, incline the tube, and, by means of a pipette allow the urine to flow slowly down the side. The liquids should stratify with the formation of a *white* zone of precipitated albumin at the point of juncture. If the albumin is present in very small amount the white zone may not form until the tube has been allowed to stand for several minutes. If the urine is quite concentrated a white zone, due to uric acid or urates, will form upon treatment with nitric acid as indicated. This ring may be easily differentiated from the albumin ring by repeating the test after diluting the urine with 3 or 4 volumes of water, whereupon the ring, if due to uric acid or urates, will not appear. It is ordinarily possible to differentiate between the albumin ring and the uric acid ring without diluting the urine, since the ring, when due to uric acid, has ordinarily a less sharply defined upper border, is generally broader than the albumin ring and frequently is situated in the urine *above* the point of contact with the nitric acid. Concentrated urines also occasionally exhibit the formation, at the point of contact, of a *crystalline* ring with very sharply defined borders. This is urea nitrate and is easily distinguished from the “fluffy” ring of albumin. If there is any difficulty in differentiation a simple dilution of the urine with water, as above described, will remove the difficulty. Various colored zones, due either to the presence of indican, bile pigments, or to the oxidation of other organic urinary constituents, may form in this test under certain conditions. These colored rings should never be confounded with the *white* ring which alone denotes the presence of albumin.

After the administration of certain drugs a white precipitate of *resin acids* may form at the point of contact of the two fluids and may cause the observer to draw wrong conclusions. This ring, if composed of resin acids, will dissolve in alcohol, whereas the albumin ring will not dissolve.

Weinberger has recently shown that a ring closely resembling the albumin ring is often obtained in urines preserved by thymol when subjected to Heller's test. The ring is due to the formation of nitrosothymol and possibly nitrothymol. If the thymol is removed from the urine by extraction with petroleum ether<sup>1</sup> previous to adding nitric acid, the ring does not form.

<sup>1</sup> Accomplished readily by gently agitating equal volumes of petroleum ether and the urine under examination for *two minutes* in a test-tube before applying the test.

An instrument called the *albumoscope* (*horismascope*) has been devised for use in this test and has met with considerable favor. The method of using the albumoscope is described below.

*Use of the Albumoscope.*—This instrument is intended to facilitate the making of “ring” tests such as Heller’s and Roberts’. In making a test about 5 c.c. of the solution under examination is first introduced into the apparatus through the larger arm and the reagent used in the particular test is then introduced through the capillary arm and allowed to flow down underneath the solution under examination. If a reasonable amount of care is taken there is no possibility of mixing the two solutions and a definitely defined white “ring” is easily obtained at the zone of contact.

2. **Roberts’ Ring Test.**—Place 5 c.c. of Roberts’ reagent<sup>1</sup> in a test-tube, incline the tube, and, by means of a pipette allow the urine to flow *slowly* down the side. The liquids should stratify with the formation of a *white* zone of precipitated albumin at the point of juncture. This test is a modification of Heller’s ring test and is rather more satisfactory than that test, since the colored rings never form and the consequent confusion is avoided. The *albumoscope* (see above) may also be used in making this test.

3. **Spiegler’s Ring Test.**—Place 5 c.c. of Spiegler’s reagent<sup>2</sup> in a test-tube, incline the tube, and, by means of a pipette, allow 5 c.c. of urine, acidified with acetic acid, to flow *slowly* down the side. A white zone will form at the point of contact. This is an exceedingly delicate test, in fact too delicate for ordinary clinical purposes, since it serves to detect albumin when present in the merest trace (1:250,000) and hence most normal urines will give a positive reaction for albumin when this test is applied.

Some investigators claim that the delicacy of this test depends upon the presence of sodium chloride in the urine, the test losing accuracy if the sodium chloride content be low.

4. **Jolles’ Reaction.**—Shake 5 c.c. of urine with 1 c.c. of 30 per cent acetic acid and 4 c.c. of Jolles’ reagent<sup>3</sup> in a test-tube. A white precipitate indicates the presence of albumin.

<sup>1</sup> Roberts’ reagent is composed of 1 volume of concentrated HNO<sub>3</sub> and 5 volumes of a saturated solution of MgSO<sub>4</sub>.

<sup>2</sup> Spiegler’s reagent has the following composition:

Tartaric acid.....	20 grams.
Mercuric chloride.....	40 grams.
Glycerol.....	100 grams.
Distilled water.....	1000 grams.

<sup>3</sup> Jolles’ reagent has the following composition:

Succinic acid.....	40 grams.
Mercuric chloride.....	20 grams.
Sodium chloride.....	20 grams.
Distilled water.....	1000 grams.



Care should be taken to use the correct amount of acetic acid, since the use of too small an amount may result in the formation of mercury combinations which may cause confusion. In the presence of iodine, mercuric iodide will form but may readily be differentiated from albumin through the fact that it is *soluble* in alcohol.

**5. Coagulation or Boiling Test.**—(a) Heat 5 c.c. of urine to boiling in a test-tube. A precipitate forming at this point is due either to albumin or to phosphates. Acidify the urine slightly by the addition of 3–5 drops of very dilute acetic acid, adding the acid drop by drop to the *hot* solution. If the precipitate is due to phosphates it will disappear under these conditions, whereas if it is due to albumin it will not only fail to disappear but will become more flocculent in character, since the reaction of a fluid must be acid to secure the complete precipitation of the albumin by this coagulation process. Too much acid should be avoided since it will cause the albumin to go into solution. Certain *resin acids* may be precipitated by the acid, but the precipitate due to this cause may be easily differentiated from the albumin precipitate by reason of its solubility in alcohol.

(b) A modification of this test in quite general use is as follows: Fill a test-tube two-thirds full of urine and gently heat the *upper half* of the fluid to boiling, being careful that this fluid does not mix with the lower half. A turbidity indicates albumin or phosphates. Acidify the urine slightly by the addition of 3–5 drops of dilute acetic acid, when the turbidity, if due to phosphates, will disappear.

Nitric acid is often used in place of acetic acid in these tests. In case nitric acid is used ordinarily 1–2 drops is sufficient.

**6. Acetic Acid and Potassium Ferrocyanide Test.**—To 5 c.c. of urine in a test-tube add 5–10 drops of acetic acid. Mix well and add potassium ferrocyanide *drop by drop*, until a precipitate forms. This is a very delicate test. Schmiedl claims that a precipitate of  $\text{Fe}(\text{Cn})_6\text{K}_2\text{Zn}$  or  $\text{Fe}(\text{Cn})_6\text{Zn}_2$  is formed when urines containing zinc are subjected to this test and that this precipitate resembles the precipitate secured with protein solutions. In the case of human urine a reaction was obtained when 0.000022 gram of zinc per cubic centimeter was present. Schmiedl further found that the urine collected from rabbits housed in zinc-lined cages possessed a zinc content which was sufficient to yield a ready response to the test. Zinc is the only interfering substance so far reported.

**7. Tanret's Test.**—To 5 c.c. of urine in a test-tube add Tarnet's reagent<sup>1</sup> drop by drop until a turbidity or precipitate forms. This

<sup>1</sup> Tanret's reagent is prepared as follows: Dissolve 1.35 gram of mercuric chloride in 25 c.c. of water, add to this solution 3.32 grams of potassium iodide dissolved in 25 c.c. of water, then make the total solution up to 60 c.c. with water and add 20 c.c. of glacial acetic acid to the mixture.

is an exceedingly delicate test. Sometimes the urine is stratified upon the reagent as in Heller's or Roberts' ring test. According to Repiton, urates interfere with the delicacy of this test. Tanret, however, claims that urates do not interfere inasmuch as any precipitate due to urates may be brought into solution by heat whereas an albumin precipitate under the same conditions will persist. Tanret further states that *mucin* interferes with the delicacy of the test and that it should therefore be removed from the urine under examination by acidification with acetic acid and filtration before testing for albumin.

**8. Sodium Chloride and Acetic Acid Test.**—Mix two volumes of urine and one volume of a saturated solution of sodium chloride in a test-tube, acidify with acetic acid, and heat to boiling. The production of a cloudiness or the formation of a precipitate indicates the presence of albumin. The resin acids may interfere here as in the ordinary coagulation test (page 335), but they may be easily differentiated from albumin by means of their solubility in alcohol.

**9. Potassium Iodide Test.**<sup>1</sup>—Dilute 5 c.c. of the urine under examination with 10 c.c. of water and stratify this mixture upon a potassium iodide solution made slightly acid with acetic acid. In the presence of 0.01–0.02 per cent of albumin a white ring forms immediately. If the test be allowed to stand two minutes after the stratification it will serve to detect 0.005 per cent of albumin.

#### GLOBULIN.

Serum globulin is not a constituent of normal urine but frequently occurs in the urine under pathological conditions and is ordinarily associated with serum albumin. In albuminuria globulin in varying amounts often accompanies the albumin, and the clinical significance of the two is very similar. Under certain conditions globulin may occur in the urine unaccompanied by albumin.

#### EXPERIMENTS.

Globulin will respond to all the tests just outlined under Albumin. If it is desirable to differentiate between albumin and globulin in any urine the following processes may be employed:

**1. Saturation with Magnesium Sulphate.**—Place 25 c.c. of neutral urine in a small beaker and add pulverized magnesium sulphate *in substance* to the point of saturation. If the protein present is globulin it will precipitate at this point. If no precipitate is produced acidify the saturated solution with acetic acid and warm gently. Albumin will be precipitated if present.

The above procedure may be used to separate globulin and albumin

<sup>1</sup> *Pharm. Zig.*, 54, p. 612.

f present in the same urine. To do this filter off the globulin after it has been precipitated by the magnesium sulphate, then acidify the clear solution and warm gently as directed. Note the formation of the albumin precipitate.

2. **Half-saturation with Ammonium Sulphate.**—Place 25 c.c. of neutral urine in a small beaker and add an equal volume of a saturated solution of ammonium sulphate. Globulin, if present, will be precipitated. If no precipitate forms add ammonium sulphate *in substance* to the point of saturation. If albumin is present it will be precipitated upon saturation of the solution as just indicated. This method may also be used to separate globulin and albumin when they occur in the same urine.

Frequently in urine which contains a large amount of urates a precipitate of ammonium urate may occur when the ammonium sulphate solution is added to the urine. This urate precipitate should not be confounded with the precipitate due to globulin. The two precipitates may be differentiated by means of the fact that the urate precipitate ordinarily appears only after the lapse of several minutes whereas the globulin generally precipitates at once.

#### PROTEOSE AND PEPTONE.

Proteoses, particularly deuteroproteose and heteroproteose, have frequently been found in the urine under various pathological conditions such as diphtheria, pneumonia, intestinal ulcer, carcinoma, dermatitis, osteomalacia, atrophy of the kidneys, and in sarcoma of the bones of the trunk. "Bence-Jones' protein," a proteose-like substance, is of interest in this connection and its appearance in the urine is believed to be of great diagnostic importance in cases of multiple myeloma or myelogenic osteosarcoma. By some investigators this protein is held to be a variety of heteroproteose whereas others claim that it possesses albumin characteristics.

Peptone certainly occurs much less frequently as a constituent of the urine than does proteose, in fact most investigators seriously question its presence under any conditions. There are many instances of peptonuria cited in the early literature, but because of the uncertainty in the conception of what really constituted a peptone it is probable that in many cases of so-called peptonuria the protein present was really proteose.

#### EXPERIMENTS.

1. **Boiling Test.**—Make the ordinary coagulation test according to the directions given under Albumin, page 335. If no coagulable

protein is found allow the boiled urine to stand and note the gradual appearance, in the cooled fluid, of a flaky precipitate of proteose. This is a crude test and should never be relied upon.

2. **Schulte's Method.**—Acidify 50 c.c. of urine with dilute acetic acid and filter off any precipitate of nucleoprotein which may form. Now test a few cubic centimeters of the urine for coagulable protein, by tests 2 and 5 under Albumin, pp. 334-5. If coagulable protein is present remove it by coagulation and filtration before proceeding. Introduce 25 c.c. of the urine, freed from coagulable protein, into 150 c.c. of absolute alcohol and allow it to stand for 12-24 hours. Decant the supernatant fluid and dissolve the precipitate in a small amount of hot water. Now filter this solution, and after testing again for nucleoprotein with *very dilute* acetic acid, try the biuret test. If this test is positive the presence of proteose is indicated.<sup>1</sup>

Urobilin does not ordinarily interfere with this test since it is almost entirely dissolved by the absolute alcohol when the proteose is precipitated.

3. **v. Aldor's Method.**—Acidify 10 c.c. of urine with hydrochloric acid, add phosphotungstic acid until no more precipitate forms and centrifugate<sup>2</sup> the solution. Decant the supernatant fluid, add some absolute alcohol to the precipitate, and centrifugate again. This washing with alcohol is intended to remove the urobilin and hence should be continued so long as the alcohol exhibits any coloration whatever. Now suspend the precipitate in water and add potassium hydroxide to bring it into solution. At this point the solution may be blue in color, in which case decolorization may be secured by gently heating. Apply the biuret test to the *cool* solution. A positive biuret test indicates the presence of proteoses.

4. **Detection of "Bence-Jones' Protein."**—Heat the suspected urine very gently, carefully noting the temperature. At as low a temperature as 40° C. a turbidity may be observed, and as the temperature is raised to about 60° C. a flocculent precipitate forms and clings to the sides of the test-tube. If the urine is now acidified *very slightly* with acetic acid and the temperature further raised to 100° C. the precipitate at least partly disappears; it will return upon cooling the tube.

This property of precipitating at so low a temperature and of dissolving at a higher temperature is typical of "Bence-Jones' protein" and may be used to differentiate it from all other forms of protein material occurring in the urine.

<sup>1</sup> If it is considered desirable to test for peptone the proteose may be removed by saturation with  $(\text{NH}_4)_2\text{SO}_4$  according to the directions given on page 120 and the filtrate tested for peptone by the biuret test.

<sup>2</sup> If not convenient to use a centrifuge the precipitate may be filtered off and washed on the filter paper with alcohol.

### NUCLEOPROTEIN.

There has been considerable controversy as to the proper classification for the protein body which forms the "nubecula" of normal urine. By different investigators it has been called *mucin*, *mucoïd*, *phosphoprotein*, *nucleoalbumin*, and *nucleoprotein*. Of course, according to the modern acceptation of the meanings of these terms they cannot be synonymous. Mucin and mucoïd are glycoproteins and hence contain no phosphorus (see p. 112), whereas phosphoproteins and nucleoproteins are phosphorized bodies. It may possibly be that both these forms of protein, *i. e.*, the glycoprotein and the phosphorized type, occur in the urine under certain conditions (see page 308). In this connection we will use the term *nucleoprotein*. The pathological conditions under which the content of nucleoprotein is increased includes all affections of the urinary passages and in particular pyelitis, nephritis, and inflammation of the bladder.

### EXPERIMENTS.

1. **Detection of Nucleoprotein.**—Place 10 c.c. of urine in a small beaker, dilute it with three volumes of water to prevent precipitation of urates, and make the reaction *very strongly* acid with acetic acid. If the urine becomes turbid it is an indication that nucleoprotein is present.

If the urine under examination contains albumin the greater portion of this substance should be removed by boiling the urine before testing it for the presence of nucleoprotein.

2. **Ott's Precipitation Test.**—Mix 25 c.c. of the urine with an equal volume of a saturated solution of sodium chloride and slowly add Almén's reagent.<sup>1</sup> In the presence of nucleoprotein a voluminous precipitate forms.

### BLOOD.

The pathological conditions in which blood occurs in the urine may be classified under the two divisions *hæmaturia* and *hæmoglobinuria*. In *hæmaturia* we are able to detect not only the hæmoglobin but the unruptured corpuscles as well, whereas in *hæmoglobinuria* the pigment alone is present. *Hæmaturia* is brought about through blood passing into the urine because of some lesion of the kidney or of the urinary tract below the kidney. *Hæmoglobinuria* is brought about through hæmolysis, *i. e.*, the rupturing of the stroma of the erythrocyte and the liberation of the hæmoglobin. This may occur in scurvy, typhus, pyemia, purpura, and in other diseases. It may also occur as the result of a burn covering a

<sup>1</sup> Dissolve 5 grams of tannin in 240 c.c. of 50 per cent alcohol and add 10 c.c. of 25 per cent acetic acid.

considerable area of the body, or may be brought about through the action of certain poisons or by the injections of various substances having the power of dissolving the erythrocytes. Transfusion of blood may also cause hæmoglobinuria.

#### EXPERIMENTS.

1. **Heller's Test.**—Render 10 c.c. of urine strongly alkaline with potassium hydroxide solution and heat to boiling. Upon allowing the heated urine to stand a precipitate of phosphates, colored red by the contained hæmatin, is formed. It is ordinarily well to make a "control" experiment using normal urine, before coming to a final decision.

Certain substances, such as cascara sagrada, rhubarb, santonin, and senna, cause the urine to give a similar reaction. Reactions due to such substances may be differentiated from the true blood reaction by the fact that both the precipitate and the pigment of the former reaction disappear when treated with acetic acid, whereas if the color is due to hæmatin the acid will only dissolve the precipitate of phosphates and leave the pigment undissolved.

2. **Teichmann's Hæmin Test.**—Place a small drop of the suspected urine or a small amount of the moist sediment on a microscopic slide, add a minute grain of sodium chloride and *carefully* evaporate to *dryness* over a *low* flame. Put a cover glass in place, run underneath it a drop of glacial acetic acid, and warm gently until the formation of gas bubbles is observed. Cool the preparation, examine under the microscope, and compare the form of the crystals with those reproduced in Figs. 59 and 60, page 211. (See Atkinson and Kendall's modification, p. 210.)

3. **Heller-Teichmann Reaction.**—Produce the pigmented precipitate according to directions given in Heller's test above. If there is a copious precipitate of phosphates and but little pigment the phosphates may be dissolved by treatment with acetic acid and the residue used in the formation of the hæmin crystals according to directions in Experiment 2, above.

4. v. **Zeynek and Nencki's Hæmin Test.**—To 10 c.c. of the urine under examination add acetone until no more precipitate forms. Filter off the precipitate and extract it with 10 c.c. of acetone rendered acid with 2-3 drops of hydrochloric acid. Place a drop of the resulting colored extract on a slide, immediately place a cover glass in position, and examine under the microscope. Compare the form of the crystals with those shown in Figs. 59 and 60, page 211. Hæmin crystals produced by this manipulation are sometimes very minute, thus rendering it difficult to determine the exact form of the crystal.

5. **Schalhjew's Hæmin Test.**—Place 20 c.c. of glacial acetic acid in

small beaker and heat to 80° C. Add 5 c.c. of the urine under examination, raise the temperature to 80° C., and stand the mixture aside to cool. Examine the crystals under the microscope and compare them with those shown in Figs. 59 and 60, page 211.

6. **Guaiac Test.**—Place 5 c.c. of urine in a test-tube and by means of a pipette introduce a freshly prepared alcoholic solution of guaiaic (strength about 1:60) into the fluid until a turbidity results, then add old turpentine or hydrogen peroxide, drop by drop, until a blue color is obtained. This is a very delicate test when properly performed. Bucknaster has recently suggested the use of guaiaconic acid instead of the solution of guaiaic. See discussion on page 204 and test on page 209.

7. **Schumm's Modification of the Guaiac Test.**—To about 5 c.c. of urine<sup>1</sup> in a test-tube add about 10 drops of a freshly prepared alcoholic solution of guaiaic. Agitate the tube gently, add about 20 drops of old turpentine, subject the tube to a thorough shaking, and permit it to stand for about 2–3 minutes. A blue color indicates the presence of blood in the solution under examination. In case there is not sufficient blood to yield a blue color under these conditions, a few c.c. of alcohol should be added and the tube gently shaken, whereupon a blue coloration will appear in the upper alcohol-turpentine layer.

A control test should always be made using water in place of urine. In the detection of very minute traces of blood only 3–5 drops of the guaiaic solution should be employed.

8. **Adler's Benzidine Reaction.**—This is one of the most delicate of the reactions for the detection of blood. Different benzidine preparations vary greatly in their sensitiveness, however. Inasmuch as benzidine solutions change readily upon contact with light, it is essential that they be kept in a dark place. The test is performed as follows: To a saturated solution of benzidine in alcohol or glacial acetic acid add an equal volume of 3 per cent hydrogen peroxide and 1 c.c. of the urine under examination. If the mixture is not already acid, render it so with acetic acid, and note the appearance of a green or blue color. A control test should be made substituting water for the urine.

Often when urines containing a small amount of blood are tested by this reaction, the mixture is rendered so turbid as to make it difficult to decide as to the presence of a faint green color. Such urines should be extracted with an ether-acetic acid solution and the resulting extract washed with water before the test is applied to it. The sensitiveness of the benzidine reaction is greater when applied to aqueous solutions than when applied to the urine.

<sup>1</sup> Alkaline urine should be made slightly acid with acetic acid as the blue end-reaction is very sensitive to alkali.

9. **Spectroscopic Examination.**—Submit the urine to a spectroscopic examination according to the directions given on page 215, looking especially for the absorption-bands of oxyhæmoglobin and methæmoglobin (see Absorption Spectra, Plate I.).

## BILE.

Both the pigments and the acids of the bile may be detected in the urine under certain pathological conditions. Of the pigments, bilirubin is the only one which has been positively identified in fresh urine; the other pigments, when present, are probably derived from the bilirubin. A urine containing bile may be yellowish-green to brown in color and when shaken foams readily. The staining of the various tissues of the body through the absorption of bile due to occlusion of the bile duct cause a condition known as icterus or jaundice. Bile is always present in the urine under such conditions unless the amount of bile reaching the tissues is extremely small.

## EXPERIMENTS.

### Tests for Bile Pigments.

1. **Gmelin's Test.**—To about 5 c.c. of *concentrated* nitric acid in a test-tube add an equal volume of urine *carefully* so that the two fluids do not mix. At the point of contact note the various colored rings, *green, blue, violet, red, and reddish-yellow.*

2. **Rosenbach's Modification of Gmelin's Test.**—Filter 5 c.c. of urine through a small filter paper. Introduce a drop of *concentrated* nitric acid into the cone of the paper and observe the succession of colors as given in Gmelin's test.

3. **Nakayama's Reaction.**—To 5 c.c. of urine in a test-tube add an equal volume of a 10 per cent solution of barium chloride. Centrifugate the mixture, pour off the supernatant fluid, and heat the precipitate with 2 c.c. of Nakayama's reagent.<sup>1</sup> In the presence of bile pigments the solution assumes a blue or green color.

3. **Huppert's Reaction.**—Thoroughly shake equal volumes of urine and milk of lime in a test-tube. The pigments unite with the calcium and are precipitated. Filter off the precipitate, wash it with water, and transfer to a small beaker. Add alcohol acidified slightly with hydrochloric acid and warm upon a water-bath until the solution becomes colored an emerald green.

According to Steensma, this procedure may give negative results

<sup>1</sup>Prepared by combining 99 c.c. of alcohol and 1 c.c. of fuming hydrochloric acid containing 4 grams of ferric chloride per liter.



even in the presence of the pigments, owing to the fact that the acid-alcohol is not a sufficiently strong oxidizing agent. He therefore suggests the addition of a drop of a 0.5 per cent solution of sodium nitrite to the acid-alcohol mixture before warming on the water-bath. Try this modification also.

4. **Salkowski's Test.**—Render 5 c.c. of urine alkaline with a few drops of a 10 per cent sodium carbonate solution and add a 10 per cent solution of calcium chloride, drop by drop, until the supernatant fluid exhibits the normal urinary color when the contents of the test-tube are thoroughly mixed. Filter off the precipitate, and after washing it place it in a second tube with 95 per cent alcohol. Acidify the alcohol with hydrochloric acid and, if necessary, shake the tube to bring the precipitate into solution. Heat the solution to boiling and observe the appearance of a green color which changes through blue and violet to red; if no bile is present the solution does not undergo any color change. This test will frequently exhibit greater delicacy than Gmelin's test. Steensma's suggestions mentioned under Huppert's Reaction, above, apply in connection with this test also.

5. **Hammarsten's Reaction.**—To about 5 c.c. of Hammarsten's reagent<sup>1</sup> in a small evaporating dish add a few drops of urine. A green color is produced. If more of the reagent is now added the play of colors as noted in Gmelin's test may be obtained.

6. **Smith's Test.**—To 2-3 c.c. of urine in a test-tube *add carefully* about 5 c.c. of dilute tincture of iodine (1:10) so that the fluids do not mix. A green ring is observed at the point of contact.

7. **Salkowski-Schippers Reaction.**—Neutralize the acidity of 10 c.c. of the urine under examination with a few drops of a dilute solution of sodium carbonate, and add 5 drops of a 20 per cent solution of sodium carbonate and 10 drops of a 20 per cent solution of calcium chloride. Filter off the resultant precipitate upon a hardened filter paper and wash it with water. Remove the precipitate to a small porcelain dish, add 3 c.c. of an acid-alcohol mixture<sup>2</sup> and a few drops of a dilute solution of sodium nitrite and heat. The production of a green color indicates the presence of bile pigments.

8. **Bonanno's Reaction.**<sup>3</sup>—Place 5-10 c.c. of the urine under examination in a small porcelain evaporating dish and add a few drops of Bonanno's reagent.<sup>4</sup> If bile is present an emerald-green color will

<sup>1</sup> Hammarsten's reagent is made by mixing 1 volume of 25 per cent nitric acid and 19 volumes of 25 per cent hydrochloric acid and then adding 1 volume of this acid mixture 0.4 volumes of 95 per cent alcohol.

<sup>2</sup> Made by adding 5 c.c. of concentrated hydrochloric acid to 95 c.c. of 96 per cent alcohol.

<sup>3</sup> Il Tommasi, 2, No. 21.

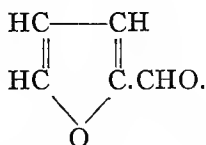
<sup>4</sup> This reagent may be prepared by dissolving 2 grams of sodium nitrite in 100 c.c. of concentrated hydrochloric acid.

develop. Bonanno says the reaction is not interfered with by any known normal or pathological urinary constituent.

#### Tests for Bile Acids.

1. **Pettenkofer's Test.**—To 5 c.c. of urine in a test-tube add 5 drops of a 5 per cent solution of sucrose. Now incline the tube, run about 2–3 c.c. of concentrated sulphuric acid *carefully* down the side and note the *red* ring at the point of contact. Upon slightly agitating the contents of the tube the whole solution gradually assumes a *reddish* color. As the tube becomes warm, it should be cooled in running water in order that the temperature may not rise above 70° C.

2. **Mylius's Modification of Pettenkofer's Test.**—To approximately 5 c.c. of urine in a test-tube add 3 drops of a very dilute (1:1000) aqueous solution of furfural,



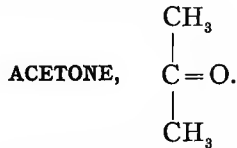
Now incline the tube, run about 2–3 c.c. of concentrated sulphuric acid carefully down the side and note the *red* ring as above. In this case also, upon shaking the tube, the whole solution is colored red. Keep the temperature below 70° C. as before.

3. **Neukomm's Modification of Pettenkofer's Test.**—To a few drops of urine in an evaporating dish add a trace of a dilute sucrose solution and one or more drops of dilute sulphuric acid. Evaporate on a water-bath and observe the development of a *violet* color at the edge of the evaporating mixture. Discontinue the evaporation as soon as the color is observed.

4. **v. Udransky's Test.**—To 5 c.c. of urine in a test-tube add 3–4 drops of a very dilute (1:1000) aqueous solution of furfural. Place the thumb over the top of the tube and shake until a thick foam is formed. By means of a small pipette add 2–3 drops of concentrated sulphuric acid to the foam and observe the *dark pink* coloration produced.

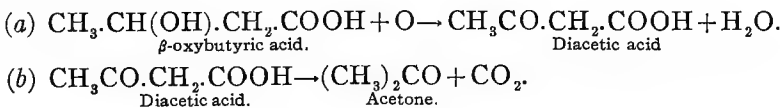
5. **Hay's Test.**—This test is based upon the principle that bile acids have the property of reducing the surface tension of fluids in which they are contained. The test is performed as follows: Cool about 10 c.c. of urine in a test-tube to 17° C. or lower, and sprinkle a little finely pulverized sulphur upon the surface of the fluid. The presence of bile acids is indicated if the sulphur sinks to the bottom of the liquid, the rapidity with which the sulphur sinks depending upon the amount of bile acids present in the urine. The test is said to react with bile acids when the latter are present in the proportion 1:120,000.

Some investigators claim that it is impossible to differentiate between bile acids and bile pigments by this test.



It was formerly very generally believed that acetone appeared in the urine under pathological conditions because of increased protein decomposition. It is now generally thought that, in man, the output of acetone arises principally from the breaking down of fatty tissues or fatty foods within the organism. The quantity of acetone eliminated has been shown to increase when the subject is fed an abundance of fat-containing food as well as during fasting, whereas a replacement of the fat with carbohydrates is followed by a marked decrease in the acetone excretion. Conditions are different with certain of the lower animals. With the dog, for instance, the output of acetone is not diminished when the animal is fed upon a carbohydrate diet, is *decreased* during fasting, and increased when the animal is fed upon a diet of meat.

Acetone and the closely related bodies,  $\beta$ -oxybutyric acid and diacetic acid, are generally classified as the *acetone bodies*. They are all associated with a deranged metabolic function and may appear in the urine together or separately, depending upon the conditions. Acetone and diacetic acid may occur alone in the urine but  $\beta$ -oxybutyric acid is never found except in conjunction with one or the other of these bodies. Acetone and diacetic acid arise chiefly from the oxidation of  $\beta$ -oxybutyric acid. The relation existing between these three bodies is shown in the following reactions:



Acetone, chemically considered, is a ketone, *di-methyl ketone*. When pure it is a liquid which possesses a characteristic aromatic fruit-like odor, boils at 56–57° C. and is miscible with water, alcohol, or ether in all proportions. Acetone is a *physiological* as well as a pathological constituent of the urine and under normal conditions the daily output is about 0.01–0.03 gram.

Pathologically, the elimination of acetone is often greatly increased and at such times a condition of *acetonuria* is said to exist. This pathological acetonuria may accompany diabetes mellitus, scarlet fever, typhoid

fever, pneumonia, nephritis, phosphorus poisoning, grave anæmias, fasting, and a deranged digestive function; it also frequently accompanies auto-intoxication and chloroform and ether anæsthesia. The types of acetonuria most frequently met with are those noted in febrile conditions and in advanced cases of diabetes mellitus.

#### EXPERIMENTS.

1. **Isolation from the Urine.**—In order to facilitate the detection of acetone in the urine, the specimen under examination should be distilled and the tests as given below applied to the resulting distillate. If it is not convenient to distil the urine, the tests may be conducted upon the undistilled fluid. To obtain an acetone distillate proceed as follows: Place 100–250 c.c. of urine in a distillation flask or retort and render it acid with acetic acid. Collect about one-third of the original volume of fluid as a distillate, add 5 drops of 10 per cent hydrochloric acid and redistil about one-half of this volume. With this final distillate conduct the tests as given below.

2. **Gunning's Iodoform Test.**—To about 5 c.c. of the urine or distillate in a test-tube add a few drops of Lugol's solution<sup>1</sup> or ordinary iodine solution (I in KI) and enough  $\text{NH}_4\text{OH}$  to form a black precipitate (nitrogen iodide). Allow the tube to stand (the length of time depending upon the content of acetone in the fluid under examination) and note the formation of a yellowish sediment consisting of iodoform. Examine the sediment under the microscope and compare the form of the crystals with those shown in Fig. 7, p. 47. If the crystals are not well formed recrystallize them from ether and examine again. The crystals of iodoform should not be confounded with those of stellar phosphate (Fig. 81, p. 242) which may be formed in this test, particularly if made upon the undistilled urine. This test is preferable to Lieben's test (4) since no substance other than acetone will produce iodoform when treated according to the directions for this test; both alcohol and aldehyde yield iodoform when tested by Lieben's test.

Gunning's test is rather the most satisfactory test yet suggested for the detection of acetone, and may be used with good results even upon the undistilled urine. In some instances where the amount of acetone present is very small it is necessary to allow the tube to stand 24 hours before making the examination for iodoform crystals. This test serves to detect acetone when present in the ratio 1 : 100,000.

3. **Legal's Test.**—Introduce about 5 c.c. of the urine or distillate into a test-tube, add a few drops of freshly prepared aqueous solution

<sup>1</sup> Lugol's solution may be prepared by dissolving 4 grams of iodine and 6 grams of potassium iodide in 100 c.c. of distilled water.

of sodium nitroprusside and render the mixture alkaline with potassium hydroxide. A ruby red color, due to creatinine, a normal urinary constituent, is produced (see Weyl's test, p. 296). Add an excess of acetic acid and if acetone is present the red color will be intensified, whereas in the absence of acetone a yellow color will result. Make a control test upon normal urine to show that this is so. A similar red color may be produced by paracresol in urines containing no acetone.

4. **Lieben's Test.**—Introduce 5 c.c. of the urine or distillate into a test-tube, render it alkaline with potassium hydroxide and add 1–2 c.c. of iodine solution drop by drop. If acetone is present a yellowish precipitate of iodoform will be produced. Identify the iodoform by means of its characteristic odor and its typical crystalline form (see Fig. 7, p. 47). While fully as delicate as Gunning's test (2) this test is not as accurate since by means of the procedure involved, either alcohol or aldehyde will yield a precipitate of iodoform. This test is especially liable to lead to erroneous deductions when urines from the advanced stages of diabetes are under examination, because of the presence of alcohol formed from the sugar through fermentative processes.<sup>1</sup>

5. **Reynolds-Gunning Test.**—This test depends upon the solubility of mercuric oxide in acetone and is performed as follows: To 5 c.c. of the urine or distillate add a few drops of mercuric chloride, render the solution alkaline with potassium hydroxide and add an equal volume of 95 per cent alcohol. Shake thoroughly in order to bring the major portion of the mercuric oxide into solution and filter. Render the clear filtrate faintly acid with hydrochloric acid and stratify some ammonium sulphide,  $(\text{NH}_4)_2\text{S}$ , upon this acid solution. At the zone of contact a blackish-gray ring of precipitated mercuric sulphide,  $\text{HgS}$ , will form. Aldehyde also responds to this test. Aldehyde, however, has never been detected in the urine and could only be present in this instance if the acidified urine was distilled too far.

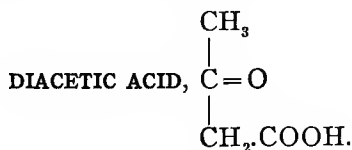
6. **Taylor's Test.**—To 10 c.c. of the urine or distillate in a test-tube add a few drops of a freshly prepared aqueous solution of sodium nitroprusside and stratify concentrated ammonium hydroxide upon the mixture. The production of a magenta color at the point of contact indicates the presence of acetone in the urine or distillate under examination. Normal urine yields an orange-red color when subjected to this technic.

**Rothera's Reaction.**<sup>2</sup>—To 5–10 c.c. of urine or distillate in a test-tube add a little solid ammonium sulphate, 2–3 drops of a freshly pre-

<sup>1</sup> Welker reports the production of a pink or red color during the application of this test to the distillates from pathological urines which had been preserved with powdered thymol. He found the color to be due to an iodothymol compound which had been previously prepared synthetically by Messinger and Vortmann.

<sup>2</sup> Rothera: *Jour. Physiol.*, 37, 491, 1908.

pared 5 per cent solution of sodium nitroprusside and 1-2 c.c. of concentrated ammonium hydroxide. The development of a *permanganate* color indicates the presence of acetone.



Diacetic or acetoacetic acid occurs in the urine only under pathological conditions and is rarely found except associated with acetone. It is formed from  $\beta$ -oxybutyric acid, another of the *acetone bodies*, and upon decomposition yields acetone and carbon dioxide. Diaceturia occurs ordinarily under the same conditions as the pathological acetonuria, *i. e.*, in fevers, diabetes, etc. (see p. 345). If very little diacetic acid is formed it may be transformed into acetone, whereas if a larger quantity is produced both acetone and diacetic acid may be present in the urine. Diaceturia is most frequently observed in children, especially accompanying fevers and digestive disorders; it is perhaps less frequently observed in adults, but when present, particularly in fevers and diabetes, it is frequently followed by fatal coma.

Diacetic acid is a colorless liquid which is miscible with water, alcohol, and ether, in all proportions. It differs from acetone in giving a violet-red or Bordeaux-red color with a dilute solution of ferric chloride.

#### EXPERIMENTS.

1. **Gerhardt's Test.**—To 5 c.c. of urine in a test-tube add ferric chloride solution, drop by drop, until no more precipitate forms. In the presence of diacetic acid a Bordeaux-red color is produced; this color may be somewhat masked by the precipitate of ferric phosphate, in which case the fluid should be filtered.

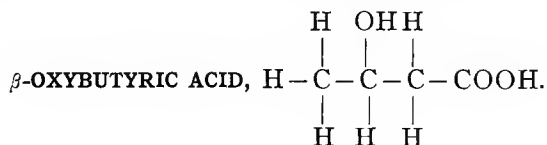
A positive result from the above manipulation simply indicates the *possible* presence of diacetic acid. Before making a final decision regarding the presence of this body make the two following control experiments:

(a) Place 5 c.c. of urine in a test-tube and boil it vigorously for 3-5 minutes. Cool the tube and, with the boiled urine, make the test as given above. As has been already stated, diacetic acid yields acetone upon decomposition and acetone does *not* give a Bordeaux-red color with ferric chloride. By boiling as indicated above, therefore, any diacetic acid present would be decomposed into acetone and carbon dioxide and the test upon the resulting fluid would be negative. If positive the color is due to the presence of bodies other than diacetic acid.

(b) Place 5 c.c. of urine in a test-tube, acidify with  $H_2SO_4$ , to free lactic acid from its salts, and *carefully* extract the mixture with ether by shaking. If diacetic acid is present it will be extracted by the ether. Now remove the ethereal solution, evaporate it to dryness, dissolve the residue in 1-2 c.c. of water and add 3-5 drops of 3 per cent ferric chloride. Diacetic acid is indicated by the production of the characteristic Bordeaux-red color. This color disappears spontaneously in 24-48 hours. Such substances as antipyrin, kairin, phenacetin, salicylic acid, salicylates, sodium acetate, thiocyanates, and thallin yield a similar red color under these conditions, but when due to the presence of any of these substances the color does not disappear spontaneously but may remain permanent for days. Many of these disturbing substances are soluble in benzene or chloroform and may be removed from the urine by this means before extracting with ether as above. Diacetic acid is insoluble in benzene or chloroform.

2. **Arnold-Lipliawsky Reaction.**—This reaction is somewhat more delicate than Gerhardt's test (1) and serves to detect diacetic acid when present in the proportion of 1:25,000. It is also negative toward acetone,  $\beta$ -oxybutyric acid and the interfering drugs mentioned as causing erroneous deductions in the application of Gerhardt's test. If the urine under examination is highly pigmented it should be partly decolorized by means of animal charcoal before applying the test as indicated below.

Place 5 c.c. of the urine under examination and an equal volume of the Arnold-Lipliawsky reagent<sup>1</sup> in a test-tube, add a few drops of concentrated ammonia and shake the tube vigorously. Note the production of a brick-red color. Take 1-2 c.c. of this colored solution, add 10-20 c.c. of hydrochloric acid (sp. gr. 1.19), 3 c.c. of chloroform, and 2-4 drops of ferric chloride solution and carefully mix the fluids. Diacetic acid is indicated by the chloroform assuming a violet or blue color; if lactic acid is absent the color may be yellow or light red.



This acid does not occur as a normal constituent of urine but is found only under pathological conditions and then always in conjunction with

<sup>1</sup> This reagent consists of two definite solutions which are ordinarily preserved separately and mixed just before using. The two solutions are prepared as follows:

(a) One per cent aqueous solution of potassium nitrite.

(b) One gram of *p*-amino-acetophenon dissolved in 100 c.c. of distilled water and enough hydrochloric acid (about 2 c.c.) added, drop by drop, to cause the solution, which is at first yellow, to become entirely colorless. An excess of acid must be avoided.

Before using, *a* and *b* are mixed in the ratio 1 : 2.

either acetone or diacetic acid. Either of these bodies may be formed from  $\beta$ -oxybutyric acid under proper conditions. It is present in especially large amount in severe cases of diabetes and has also been detected in digestive disturbances, continued fevers, scurvy, measles, and in starvation. It is probable that, in man,  $\beta$ -oxybutyric acid, in common with acetone and diacetic acid, arises principally from the breaking down of fatty tissues within the organism. The condition in which large amounts of acetone and diacetic acid, and in severe cases  $\beta$ -oxybutyric acid also, are excreted in the urine is known as "acidosis." In diabetes the deranged metabolic conditions cause the production of great quantities of these substances which lead to an acid intoxication and ultimately to diabetic coma.

Ordinarily  $\beta$ -oxybutyric acid is an odorless, transparent syrup, which is lævrotatory and easily soluble in water, alcohol, and ether; it may be obtained in crystalline form.

#### EXPERIMENTS.

I. **Black's Reaction.**—Inasmuch as the urinary pigments as well as any contained sugar or diacetic acid will interfere with the delicacy of this test when applied to the urine directly the following preliminary procedure is necessary: Concentrate 10 c.c. of the urine under examination to one-third or one-fourth of its original volume in an evaporating dish at a *gentle heat*. Acidify the residue with a few drops of concentrated hydrochloric acid, add sufficient plaster of Paris to make a thick paste and allow the mixture to stand until it begins to "set." It should now be stirred and broken up in the dish by means of a stirring rod with a blunt end. Extract the porous meal thus produced twice with ether by stirring and decantation. Any  $\beta$ -oxybutyric acid present will be extracted by the ether. Evaporate the ether extract spontaneously or on a water-bath, dissolve the residue in water, and neutralize it with barium carbonate. To 5 to 10 c.c. of this neutral fluid in a test-tube add two to three drops of ordinary commercial acid hydrogen peroxide. Mix by shaking and add a few drops of Black's reagent.<sup>1</sup> Permit the tube to stand and note the gradual development of a *rose color* which increases to its maximum intensity and then gradually fades.<sup>2</sup>

In carrying out the test care should be taken to see that the solution is *cold* and *approximately neutral* and that a *large excess* of hydrogen peroxide and Black's reagent are not added. In case but little  $\beta$ -oxybutyric acid is present the color will fail to appear or will be but transitory if the oxidizing

<sup>1</sup> Made by dissolving 5 grams of ferric chloride and 0.4 gram of ferrous chloride in 100 c.c. of water.

<sup>2</sup> This disappearance of color is due to the further oxidation of the diacetic acid.



agents are added in *too great excess*. It is preferable to add a few drops of the reagent and at intervals of a few minutes repeat the process until the color undergoes no further increase in intensity. One part of  $\beta$ -oxybutyric acid in 10,000 parts of the solution may be detected by this test.

2. **Polariscopic Examination.**—Subject some of the urine (free from protein) to the ordinary fermentation test (see page 331). This will remove dextrose and lævulose, which would interfere with the polariscopic test. Now examine the fermented fluid in the polariscope and if it is lævorotatory the presence of  $\beta$ -oxybutyric acid is indicated. This test is not absolutely reliable, however, since conjugate glycuronates are also lævorotatory after fermentation.

3. **Kulz's Test.**—Evaporate the urine, after fermenting it as indicated in the last test, to a syrup, add an equal volume of concentrated sulphuric acid, and distil the mixture directly without cooling. Under these conditions  $\alpha$ -crotonic acid is formed and is present in the distillate. Allow the distillate to cool slowly and note the formation of crystals of  $\alpha$ -crotonic acid which are soluble in ether and melt at  $72^{\circ}$  C. In case very slight traces of  $\beta$ -oxybutyric acid be present in the urine under examination the amount of  $\alpha$ -crotonic acid formed may be too small to yield a crystalline product. In this event the distillate should be extracted with ether, the ethereal extract evaporated, and the residue washed with water. Under these conditions the impurities will be removed and the  $\alpha$ -crotonic acid will remain behind as a residue. The melting-point of this residue may then be determined.

## CONJUGATE GLYCURONATES.

Glycuronic acid does not occur free in the urine, but is found, for the most part, in combination with phenol. Much smaller quantities are excreted in combination with indoxyl and skatoxyl. The total content of conjugate glycuronates seldom exceeds 0.004 per cent under normal conditions. The output may be very greatly increased as the result of the administration of antipyrin, borneol, camphor, chloral, menthol, morphine, naphthol, turpentine, etc. The glycuronates as a group are lævorotatory whereas glycuronic acid is dextro-rotatory. Most of the glycuronates, reduce alkaline metallic oxides and so introduce an error in the examination of urine for sugar. Conjugate glycuronates often occur associated with dextrose in glycosuria, diabetes mellitus, and in some other disorders. As a class the glycuronates are non-fermentable.

## EXPERIMENTS.

1. **Fermentation-Reduction Test.**—Test the urine by Fehling's test. If there is reduction try Barfoed's test. If negative this indicates

the absence of monosaccharides. A negative fermentation test would now indicate the presence of conjugate glycuronates (or lactose in rare cases).<sup>1</sup>

If dextrose is present in the urine tested for glycuronates the urine must first be subjected to a polariscopic examination, then fermented and a second polariscopic examination made. The sugar being dextro-rotatory and fermentable and the glycuronates being lævorotatory and non-fermentable the second polariscopic test will show a lævorotation indicative of conjugate glycuronates.

2. **Tollens' Reaction.**—Make this test according to directions given under Pentoses, p. 353.

## PENTOSSES.

We have two distinct types of pentosuria, *i. e.*, *alimentary pentosuria*, resulting from the ingestion of large quantities of pentose-rich vegetables such as prunes, cherries, grapes, or plums, and fruit juices, in which condition the pentoses appear only *temporarily* in the urine; and the *chronic* form of pentosuria, in which the output of pentoses bears no relation whatever to the quantity and nature of the pentose content of the food eaten. In occurring in these two forms, pentosuria resembles glycosuria (see page 324), but it is definitely known that pentosuria bears no relation to diabetes mellitus and there is no generally accepted theory to account for the occurrence of the chronic form of pentosuria. The pentose detected most frequently in the urine is arabinose, the inactive form generally occurring in chronic pentosuria and the lævorotatory variety occurring in the alimentary type of the disorder.

## EXPERIMENTS.

I. **Bial's Reaction.**<sup>2</sup>—To 5 c.c. of Bial's reagent<sup>3</sup> in a test-tube add 2–3 c.c. of urine and heat the mixture gently until the first bubbles rise to the surface.<sup>4</sup> Immediately or upon cooling the solution becomes green and a flocculent precipitate of the same color may form.

This test is believed to be more accurate than the orcinol test. It is claimed that urines containing *menthol*, *kreosotal*, etc., respond to the orcinol reaction, but not to Bial's.

<sup>1</sup> If necessary to differentiate between lactose and glycuronates apply the mucic acid test (see p. 354) or the phenylhydrazine reaction (see p. 28).

<sup>2</sup> Bial: *Deut. med. Woch.*, 28, 252, 1902.

<sup>3</sup> Orcinol..... 1.5 gram.

Fuming HCl..... 500 grams.

Ferric chloride (10 per cent)..... 20–30 drops.

<sup>4</sup> The test may also be performed by adding the urine to the *hot* reagent. No further heating should be necessary if pentose is present.

2. **Tollens' Reaction.**—To equal volumes of urine and hydrochloric acid (sp. gr. 1.09) add a little phloroglucinol and heat the mixture on a boiling water-bath. Pentose, galactose, or glycuronic acid will be indicated by the appearance of a red color. To differentiate between these bodies examine by the spectroscope and look for the absorption band between D and E given by pentoses and glycuronic acid, and then differentiate between the two latter bodies by the melting-points of their osazones.

3. **Orcinol Test.**—Place equal volumes of urine and hydrochloric acid (sp. gr. 1.09) in a test-tube, add a small amount of orcinol, and heat the mixture to boiling. Color changes from red through reddish-blue to green will be noted. When the solution becomes green it should be shaken in a separatory funnel with a little amyl alcohol, and the alcoholic extract examined spectroscopically. An absorption band between C and D will be observed.

### FAT.

When fat finds its way into the urine through a lesion which brings some portion of the urinary passages into communication with the lymphatic system a condition known as *chyluria* is established. The turbid or milky appearance of such urine is due to its content of chyle. This disease is encountered most frequently in tropical countries, but is not entirely unknown in more temperate climates. Albumin is a constant constituent of the urine in chyluria. Upon shaking a chylous urine with ether the fat is dissolved by the ether and the urine becomes clearer or entirely clear.

### HÆMATOPORPHYRIN.

Urine containing this body is occasionally met with in various diseases, but more frequently after the use of quinine, tetronal, trional, and especially sulphonal. Such urines ordinarily possess a reddish tint, the depth of color varying greatly under different conditions.

### EXPERIMENTS.

1. **Spectroscopic Examination.**—To 100 c.c. of urine add about 20 c.c. of a 10 per cent solution of potassium hydroxide or ammonium hydroxide. The precipitate which forms consists principally of earthy phosphates to which the hæmatoporphyrin adheres and is carried down. Filter off the precipitate, wash it and transfer to a flask and warm with

alcohol acidified with hydrochloric acid. By this process the hæmatoporphyrin is dissolved and on filtering will be found in the filtrate and may be identified by means of the spectroscope (see page 219, and Absorption Spectra, Plate II).

2. **Acetic Acid Test.**—To 100 c.c. of urine add 5 c.c. of glacial acetic acid and allow the mixture to stand 48 hours. Hæmatoporphyrin deposits in the form of a precipitate.

## LACTOSE.

Lactose is rarely found in the urine except as it is excreted by women during pregnancy, during the nursing period, or soon after weaning. It is rather difficult to show the presence of lactose in the urine in a satisfactory manner, since the formation of the characteristic lactosazone is not attended with any great measure of success under these conditions. It is, however, comparatively easy to show that it is not dextrose, for, while it responds to reduction tests, it does not ferment with *pure* yeast and does not give a dextrosazone. An absolutely conclusive test, of course, is the isolation of the lactose in crystalline form (Fig. 80, p. 238) from the urine.

On oxidation with nitric acid lactose and galactose yield *mucic acid*. This test is frequently used in urine examination to differentiate lactose and galactose from other reducing sugars.

## EXPERIMENTS.

1. **Mucic Acid Test.**—Treat 100 c.c. of the urine under examination with 20 c.c.<sup>1</sup> of concentrated nitric acid and evaporate the mixture in a broad, shallow glass vessel, upon a boiling water-bath until the volume of the solution is only about 20 c.c. At this point the fluid should be clear and a fine white precipitate of *mucic acid* should separate. If the percentage of lactose in the urine is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will form. It is impossible to differentiate between galactose and lactose by means of this test, but the reaction does serve to differentiate these two sugars from all other reducing sugars. A satisfactory differentiation between lactose and galactose may be made by means of Barfoed's test, p. 331.

2. **Rubner's Test.**—To 10 c.c. of urine in a small beaker add some lead acetate, in substance, heat to boiling, and add  $\text{NH}_4\text{OH}$  until no

<sup>1</sup> If the specific gravity of the urine is 1020 or over it is necessary to use 25-35 c.c. of nitric acid. Under these conditions the mixture should be evaporated until the remaining volume is approximately equivalent to that of the nitric acid added.

more precipitate is dissolved. In the presence of lactose a brick-red or rose-red color develops, whereas dextrose gives a coffee-brown color, maltose a light yellow color, and lævulose no color at all under the same conditions.

3. **Compound Test.**—Try the phenylhydrazine test, the fermentation test, and Barfoed's test according to directions given under Dextrose, pages 324, and 331. If these are negative, try Nylander's test, page 330. If this last test is positive, the presence of lactose is indicated.

### GALACTOSE.

Galactose has occasionally been detected in the urine, and in particular in that of nursing infants afflicted with a deranged digestive function. Lactose and galactose may be differentiated from other reducing sugars which may be present in the urine by means of the mucic acid test. This test simply consists in the production of mucic acid through oxidation of the sugar with nitric acid.

### EXPERIMENTS.

1. **Mucic Acid Test.**—Treat 100 c.c. of the urine under examination with 20 c.c.<sup>1</sup> of concentrated nitric acid and evaporate the mixture in a broad, shallow glass vessel, upon a boiling water-bath, until the volume of the solution is only about 20 c.c. At this point the fluid should be clear and a fine, white precipitate of *mucic acid* should separate. If the percentage of galactose present in the urine is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will form. It is impossible to differentiate between galactose and lactose by means of this test, but the reaction does serve to differentiate these two sugars from all other reducing sugars. A satisfactory differentiation between galactose and lactose may be made by Barfoed's test, p. 331.

2. **Tollens' Reaction.**—To equal volumes of the urine and hydrochloric acid (sp. gr. 1.09) add a little phloroglucinol and heat the mixture on a boiling water-bath. Galactose, pentose, and glycuronic acid will be indicated by the appearance of a red color. Galactose may be differentiated from the two latter substances in that its solutions exhibit no absorption bands upon spectroscopical examination.

### LÆVULOSE.

Diabetic urine frequently possesses the power of rotating the plane of polarized light to the left, thus indicating the presence of a lævorotatory,

<sup>1</sup> If the specific gravity of the urine is 1020 or over it is necessary to use 25-35 c.c. of nitric acid. Under these conditions the mixture should be evaporated until the remaining volume is approximately equivalent to that of the nitric acid added.

substance. The lævorotation is sometimes due to the presence of lævulose, although not necessarily confined to this carbohydrate, since conjugate glycuronates and  $\beta$ -oxybutyric acid, two other lævorotatory bodies, are frequently found in the urine of diabetics. Lævulose is invariably accompanied by dextrose in diabetic urine, but *lævulosuria* has been observed as a separate anomaly. The presence of lævulose may be inferred when the percentage of sugar, as determined by the titration method, is greater than the percentage indicated by the polariscopic examination.

#### EXPERIMENTS.

1. **Borchardt's Reaction.**—To about 5 c.c. of urine in a test-tube add an equal volume of 25 per cent hydrochloric acid and a few crystals of resorcinol. Heat to boiling and after the production of a red color, cool the tube under running water and transfer to an evaporating dish or beaker. Make the mixture slightly alkaline with *solid* potassium hydroxide, return it to a test-tube, add 2–3 c.c. of acetic ether, and shake the tube vigorously. In the presence of lævulose the acetic ether is colored *yellow*.

The only urinary constituents which interfere with the test are *nitrites* and *indican* and these interfere only when they are *simultaneously present*. Under these conditions, the urine should be acidified with acetic acid and heated to boiling for one minute to remove the nitrites. In case the indican content is *very large*, it will impart a *blue* color to the acetic ether, thus masking the yellow color due to lævulose. When such urines are to be examined, the indican should first be removed by Obermayer's test (see p. 299). The chloroform should then be discarded, the acid-urine mixture diluted with one-third its volume of water, and the test applied as described above. The urine of patients who have ingested *santonin* or *rhubarb* respond to the test. The test will serve to detect lævulose when present in a dilution of 1:2000, *i. e.*, 0.05 per cent.

2. **Seliwanoff's Reaction.**—To 5 c.c. of Seliwanoff's reagent<sup>1</sup> in a test-tube add a few drops of the urine under examination and heat the mixture to boiling. The presence of lævulose is indicated by the production of a red color and the separation of a red precipitate. The latter may be dissolved in alcohol to which it will impart a striking red color.

If the boiling be *prolonged* a similar reaction may be obtained with urines containing dextrose. This has been explained<sup>2</sup> in the case of dextrose as due to the transformation of the dextrose into lævulose by the catalytic action of the hydrochloric acid. The precautions necessary for

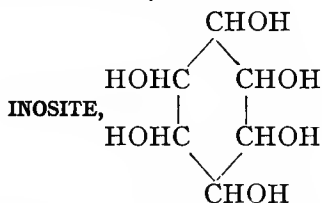
<sup>1</sup> Seliwanoff's reagent may be prepared by dissolving 0.05 gram of resorcinol in 100 c.c. of dilute (1:2) hydrochloric acid.

<sup>2</sup> Koëniçfeld: *Bioch. Zeit.*, 38, 311, 1912.

a positive test for lævulose are as follows: The concentration of the hydrochloric acid must not be more than 12 per cent. The reaction (red color) and the precipitate must be observed after not more than 20–30 seconds of boiling. Dextrose must not be present in amounts exceeding 2 per cent. The precipitate must be soluble in alcohol with a bright red color.

3. **Phenylhydrazine Test.**—Make the test according to directions under Dextrose, 3, page 324.

4. **Polariscopic Examination.**—A simple polariscopic examination, when taken in connection with other ordinary tests, will furnish the requisite data regarding the presence of lævulose, provided lævulose is not accompanied by other lævorotatory substances, such as conjugate glycuronates and  $\beta$ -oxybutyric acid.



Inosite occasionally occurs in the urine in albuminuria, diabetes mellitus, and diabetes insipidus. It is claimed also that copious water-drinking causes this substance to appear in the urine. Inosite was at one time considered to be a sugar but is now known to be hexahydroxybenzene, as the above formula indicates. It is an example of a non-carbohydrate in whose molecule the H and O are present in the proportion to form water. In other words it has the formula of the hexoses, *i. e.*,  $C_6H_{12}O_6$ . Inosite occurs widely distributed in the vegetable kingdom, and because of this fact the theory has been voiced that it represents one of the first stages in the conversion of a carbohydrate into the benzene ring. It is found in the liver, spleen, lungs, brain, kidneys, suprarenal capsules, muscles, leucocytes, testes, and urine under normal conditions.

#### EXPERIMENT.

1. **Detection of Inosite.**—Acidify the urine with concentrated nitric acid and evaporate nearly to dryness. Add a few drops of ammonium hydroxide and a little calcium chloride solution to the moist residue and evaporate the mixture to dryness. In the presence of inosite (0.001 gram) a bright red color is obtained.

For a more satisfactory test, which is also more time-consuming, see Salkowski's<sup>1</sup> modification of Scherer's test.

<sup>1</sup> Salkowski: *Zeit. physiol. chem.*, 69, 478, 1910.

### LAIOSE.

This substance is *occasionally* found in the urine in severe cases of diabetes mellitus. By some investigators laiiose is classed with the sugars. It resembles lævulose in that it has the property of reducing certain metallic oxides and is lævorotatory, but differs from lævulose in being amorphous, non-fermentable, and in not possessing a sweet taste.

### MELANINS.

These pigments never occur normally in the urine, but are present under certain pathological conditions, their presence being especially associated with melanotic tumors. Ordinarily the freshly passed urine is clear, but upon exposure to the air the color deepens and may at last be very dark brown or black in color. The pigment is probably present in the form of a chromogen or melanogen and upon coming in contact with the air oxidation occurs, causing the transformation of the melanogen into melanin and consequently the darkening of the urine.

It is claimed that melanuria is proof of the formation of a visceral melanotic growth. In many instances, without doubt, urines rich in indican have been wrongly taken as diagnostic proof of melanuria. The pigment melanin is sometimes mistaken for indigo and melanogen for indican. It is comparatively easy to differentiate between indigo and melanin through the solubility of the former in chloroform.

In rare cases melanin is found in urinary sediment in the form of fine amorphous granules.

### EXPERIMENTS.

1. **Zeller's Test.**—To 50 c.c. of urine in a small beaker add an equal volume of bromine water. In the presence of melanin a yellow precipitate will form and will gradually darken in color, ultimately becoming black.

2. **von Jaksch-Pollak Reaction.**—Add a few drops of ferric chloride solution to 10 c.c. of urine in a test-tube and note the formation of a gray color. Upon the further addition of the chloride a dark precipitate forms, consisting of phosphates and adhering melanin. An excess of ferric chloride causes the precipitate to dissolve.

This is the most satisfactory test for the identification of melanin in the urine.

### UROROSEIN.

This is a pigment which is not present in normal urine but may be detected in the urine of various diseases, such as pulmonary tuber-



culosis, typhoid fever, nephritis, and stomach disorders. Urorosein, in common with various other pigments, does not occur preformed in the urine, but is present in the form of a chromogen, which is transformed into the pigment upon treatment with a mineral acid.

#### EXPERIMENTS.

1. **Robin's Reaction.**—Acidify 10 c.c. of urine with about 15 drops of concentrated hydrochloric acid. Upon allowing the acidified urine to stand, a rose-red color will appear if urorosein is present.

2. **Nencki and Sieber's Reaction.**—To 100 c.c. of urine in a beaker add 10 c.c. of 25 per cent sulphuric acid. Allow the acidified urine to stand and note the appearance of a rose-red color. The pigment may be separated by extraction with amyl alcohol.

#### UNKNOWN SUBSTANCES.

**Ehrlich's Diazo Reaction.**—Place equal volumes of urine and Ehrlich's diazobenzenesulphonic acid reagent<sup>1</sup> in a test-tube, mix thoroughly by shaking, and quickly add ammonium hydroxide in excess. The test is positive if both the fluid and the foam assume a red color. If the tube is allowed to stand a precipitate forms, the upper portion of which exhibits a blue, green, greenish-black, or violet color. Normal urine gives a brownish-yellow reaction with the above manipulation.

The exact nature of the substance or substances upon whose presence in the urine this reaction depends is not well understood. Some investigators claim that a positive reaction indicates an abnormal decomposition of protein material, whereas others assume it to be due to an increased excretion of alloxypoteic acid, oxypoteic acid, or uroferric acid.

The reaction may be taken as a metabolic symptom of certain disorders, which is of value diagnostically *only* when taken in connection with the other symptoms. The reaction appears principally in the urine in febrile disorders and in particular in the urine in typhoid fever, tuberculosis, and measles. The reaction has also been obtained in the urine in various other disorders such as carcinoma, chronic rheumatism,

<sup>1</sup> Two separate solutions should be prepared and mixed in definite proportions when needed for use.

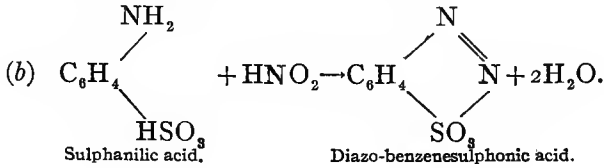
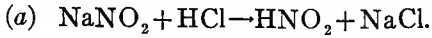
(a) Five grams of sodium nitrite dissolved in 1 liter of distilled water.

(b) Five grams of sulphanilic acid and 50 c.c. of hydrochloric acid in 1 liter of distilled water.

Solutions *a* and *b* should be preserved in well-stoppered vessels and mixed in the proportion 1 : 50 when required. Green asserts that greater delicacy is secured by mixing the solutions in the proportion 1 : 100. The sodium nitrite deteriorates upon standing and becomes unfit for use in the course of a few weeks.

diphtheria, erysipelas, pleurisy, pneumonia, scarlet fever, syphilis, typhus, etc. The administration of alcohol, chrysarobin, creosote, cresol, dionin, guaiacol, heroin, morphine, naphthalene, opium, phenol, tannic acid, etc., will also cause the urine to give a positive reaction.

The following chemical reactions take place in this test:



## CHAPTER XX.

### URINE: ORGANIZED AND UNORGANIZED SEDIMENTS.

The data obtained from carefully conducted microscopical examinations of the sediment of certain pathological urines are of very great importance, diagnostically. Too little emphasis is sometimes placed upon the value of such findings.



FIG. 102.—THE PURDY ELECTRIC CENTRIFUGE.



FIG. 103.—SEDIMENT TUBE FOR THE  
PURDY ELECTRIC CENTRIFUGE.

The sedimentary constituents may be divided into two classes, *i. e.*, *organized* and *unorganized*. The sediment is ordinarily collected for examination by means of the centrifuge (Fig. 102, above). An older method, and one still in vogue in some quarters, is the so-called *gravity* method. This simply consists in placing the urine in a conical glass and allowing the sediment to settle. The collection of the sediment by means of the centrifuge, however, is much preferable, since the process of sedimentation may be accomplished by the use of this instrument in a few minutes, and far more perfectly, whereas when the other method is

used it is frequently necessary to allow the urine to remain in the conical glass 12–24 hours before sufficient sediment can be secured for the microscopical examination.

(a) **Unorganized Sediments.**

Ammonium magnesium phosphate (“Triple phosphate”).

Calcium oxalate.

Calcium carbonate.

Calcium phosphate.

Calcium sulphate.

Uric acid.

Urates.

Cystine.

Cholesterol.

Hippuric acid.

Leucine (?) and tyrosine.

Hæmatoidin and bilirubin.

Magnesium phosphate.

Indigo.

Xanthine.

Melanin.

**Ammonium Magnesium Phosphate (“Triple Phosphate”).**—

Crystals of “triple phosphate” are a characteristic constituent of the sediment when alkaline fermentation of the urine has taken place either *before* or after being voided. They may even be detected in amphoteric or *slightly* acid urine provided the ammonium salts are present in large enough quantity. This substance may occur in the sediment in two forms, *i. e.*, prisms and the feathery type. The prismatic form of crystals (Fig. 101, p. 319) is the one most commonly observed in the sediment; the feathery form (Fig. 101, p. 319) predominates when the urine is made ammoniacal with ammonia.

The sediment of the urine in such disorders as are accompanied by a retention of urine in the lower urinary tract contains “triple phosphate” crystals as a characteristic constituent. The crystals are frequently abundant in the sediment during paraplegia, chronic cystitis, enlarged prostate, and chronic pyelitis.

**Calcium Oxalate.**—Calcium oxalate is found in the urine in the form of at least two distinct types of crystals, *i. e.*, the *dumb-bell* type and the *octahedral type* (Fig. 104, p. 363). Either form may occur in the sediment of neutral, alkaline, or acid urine, but both forms are found most frequently in urine having an acid reaction. Occasionally, in alkaline urine, the octahedral form is confounded with “triple phosphate” crystals. They

may be differentiated from the phosphate crystals by the fact that they are insoluble in acetic acid.

The presence of calcium oxalate in the urine is not of itself a sign of any abnormality, since it is a constituent of normal urine. It is increased above the normal, however, in such pathological conditions as diabetes mellitus,



FIG. 104.—CALCIUM OXALATE. (*Ogden.*)

in organic diseases of the liver, and in various other conditions which are accompanied by a derangement of digestion or of the oxidation mechanism, such as occurs in certain diseases of the heart and lungs.

**Calcium Carbonate.**—Calcium carbonate crystals form a typical constituent of the urine of herbivorous animals. They occur less fre-

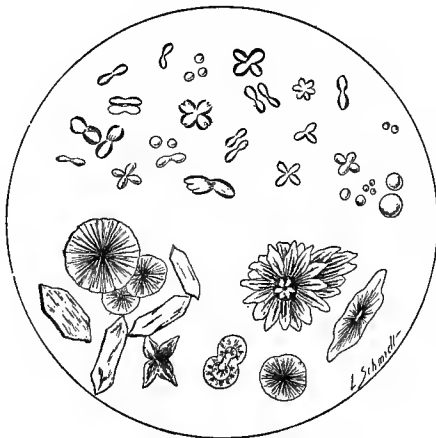


FIG. 105.—CALCIUM CARBONATE.

quently in human urine. The reaction of urine containing these crystals is nearly always alkaline, although they may occur in amphoteric or in *slightly* acid urine. It generally crystallizes in the form of granules, spherules, or dumb-bells (Fig. 105, above). The crystals of calcium carbonate may be differentiated from calcium oxalate by the fact that they dissolve in acetic acid with the evolution of carbon dioxide gas.

**Calcium Phosphate (Stellar Phosphate).**—Calcium phosphate may occur in the urine in three forms, *i. e.*, amorphous, granular, or crystalline. The crystals of calcium phosphate are ordinarily pointed, wedge-shaped formations which may occur as individual crystals, or grouped together in more or less regularly formed rosettes (Fig. 81, p. 242). Acid sodium urate crystals (Fig. 107, p. 366) are often mistaken for crystals of calcium phosphate. We may differentiate between these two crystalline forms by the fact that acetic acid will readily dissolve the phosphate, whereas the urate is much less soluble and when finally brought into solution and re-crystallized one is frequently enabled to identify uric acid crystals which have been formed from the acid urate solution. The clinical significance of the occurrence of calcium phosphate crystals in the urinary sediment is similar to that of "triple phosphate" (see page 319).

**Calcium Sulphate.**—Crystals of calcium sulphate are of quite rare occurrence in the sediment of urine. Their presence seems to be limited in general to urines which are of a decided acid reaction. Ordinarily it crystallizes in the form of long, thin, colorless needles or prisms (Fig. 100, page 316) which may be mistaken for calcium phosphate crystals. There need be no confusion in this respect, however, since the sulphate crystals are insoluble in acetic acid, which reagent readily dissolves the phosphate. As far as is known their occurrence as a constituent of urinary sediment is of very little clinical significance.

**Uric Acid.**—Uric acid forms a very common constituent of the sediment of urines which are acid in reaction. It occurs in more varied forms than any of the other crystalline sediments (Plate V, opposite page 291, and Fig. 106, page 365), some of the more common varieties of crystals being rhombic prisms, wedges, dumb-bells, whetstones, prismatic rosettes, irregular or hexagonal plates, etc. Crystals of pure uric acid are always colorless (Fig. 94, page 293), but the form occurring in urinary sediments is impure and under the microscope appears pigmented, the depth of color varying from light yellow to a dark reddish-brown according to the size and form of the crystal.

The presence of a considerable uric acid sediment does not, of necessity, indicate a pathological condition or a urine of increased uric acid content, since this substance very often occurs as a sediment in urines whose uric acid content is diminished from the normal merely as a result of changes in reaction, etc. Pathologically, uric acid sediments occur in gout, acute febrile conditions, chronic interstitial nephritis, etc. If the microscopical examination is not conclusive, uric acid may be differentiated from other crystalline urinary sediments from the fact that it is soluble in alkalis, alkali carbonates, boiling glycerol, concentrated sulphuric acid, and in certain organic bases such as ethylamine and piperidin. It also responds



PLATE VI.



AMMONIUM URATES, SHOWING SPHERULES AND THORN-APPLE-SHAPED CRYSTALS.  
(From *Ogden*, after *Peyer*.)



to the murexide test (see page 292), Schiff's reaction (see page 293) and to Moreigne's reaction (see p. 293).

**Urates.**—The urate sediment may consist of a mixture of the urates of ammonium, calcium, magnesium, potassium, and sodium. The ammonium urate may occur in neutral, alkaline, or acid urine, whereas the other forms of urates are confined to the sediments of acid urines. Sodium urate occurs in sediments more abundantly than the other urates. There are two sodium urates, the *mono* and the *di*, which may be expressed thus:  $\text{Na}^+ \text{H}^+ \rangle \text{C}_5\text{H}_2\text{N}_4\text{O}_3$ , and  $\text{Na}^+ \text{Na}^+ \rangle \text{C}_5\text{H}_2\text{N}_4\text{O}_3$ . Both salts dissociate with the production of an alkaline reaction, the alkalinity being stronger in the

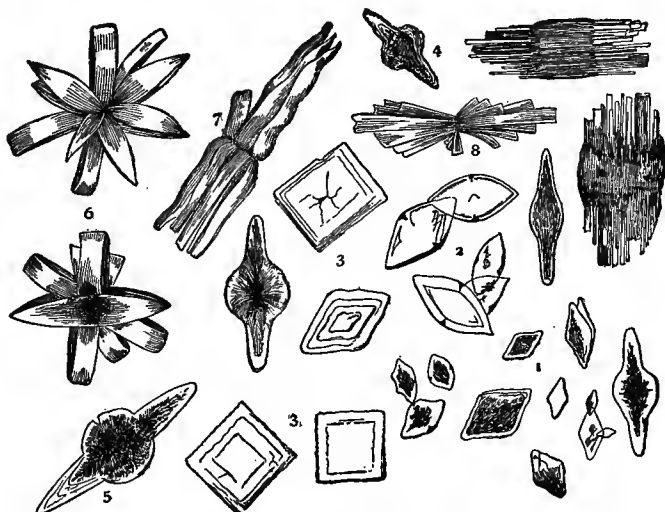


FIG. 106.—VARIOUS FORMS OF URIC ACID.

1, Rhombic plates; 2, whetstone forms; 3, 3, quadrate forms; 4, 5, prolonged into points; 6, 8, rosettes; 7, pointed bundles; 9, barrel forms precipitated by adding hydrochloric acid to urine.

case of the di-sodium urate. The so-called *quadriurate* or *hemiurate* have no existence as chemical units.<sup>1</sup> The urates of calcium, magnesium, and potassium are amorphous in character, whereas the urate of ammonium is crystalline. Sodium urate may be either amorphous or crystalline. When crystalline it forms groups of fan-shaped clusters or colorless, prismatic needles (Fig. 107, p. 366). Ammonium urate is ordinarily present in the sediment in the burr-like form of the "thorn-apple" crystal, *i. e.*, yellow or reddish-brown spheres, covered with sharp spicules or prisms (Plate VI, opposite). The urates are all soluble in hydrochloric acid or acetic acid and their acid solutions yield crystals of uric acid upon standing. They also respond to the murexide test. The clinical signifi-

Taylor: *Jour. Biol. Chem.*, 1, 177, 1905.

cance of urate sediments is very similar to that of uric acid. A considerable sediment of amorphous urates does not necessarily indicate a high uric acid content, but ordinarily signifies a concentrated urine having a very strong acidity.

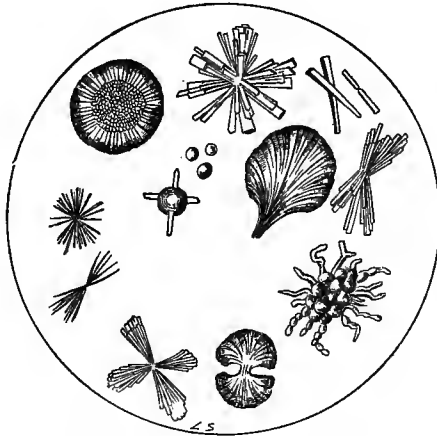


FIG. 107.—ACID SODIUM URATE.

**Cystine.**—Cystine is one of the rarer of the crystalline urinary sediments. It has been claimed that it occurs more often in the urine of men than of women. Cystine crystallizes in the form of thin, colorless, hexagonal plates (Fig. 25, p. 81, and Fig. 108, below) which are insoluble in water, alcohol, and acetic acid, and soluble in mineral acids, alkalis, and especially in ammonia. Cystine may be identified by burning it upon



FIG. 108.—CYSTINE. (Ogden.)

platinum foil, under which condition it does not melt but yields a bluish-green flame.

**Cholesterol.**—Cholesterol crystals have been but rarely detected in urinary sediments. When present they probably arise from a pathological condition of some portion of the urinary tract. Crystals of cholesterol have been found in the sediment in cystitis, pyelitis, chyluria, and nephritis.

Ordinarily it crystallizes in large regular and irregular colorless, transparent plates, some of which possess notched corners (Fig. 43, page 166). Frequently, instead of occurring in the sediment, it is found in the form of a film on the surface of the urine.

**Hippuric Acid.**—This is one of the rarer sediments of human urine. It deposits under conditions similar to those which govern the formation of uric acid sediments. The crystals, which are colorless needles or prisms (Fig. 97, page 300) when pure, are invariably pigmented in a manner similar to the uric acid crystals when observed in urinary sediment and because of this fact are frequently confounded with the rarer forms of uric acid. Hippuric acid may be differentiated from uric acid from the fact that it does not respond to the murexide test and is much more soluble in water and in ether. The detection of crystals of hippuric acid in the urine has very little clinical significance, since its presence in the sediment depends in most instances very greatly upon the nature of the diet. It is particularly prone to occur in the sediment after the ingestion of certain fruits as well as after the ingestion of benzoic acid (see page 300).

**Leucine and Tyrosine.**—Leucine and tyrosine have frequently been detected in the urine, either in solution or as a sediment. Neither of them occurs in the urine ordinarily except in association with the other, *i. e.*, whenever leucine is detected it is more than probable that tyrosine accompanies it. They have been found pathologically in the urine in acute yellow atrophy of the liver, in acute phosphorus poisoning, in cirrhosis of the liver, in severe cases of typhoid fever and small-pox, and in leukæmia. In urinary sediments leucine ordinarily crystallizes in characteristic spherical masses which show both radial and concentric striations and are highly refractive (Fig. 109, above). Some investigators claim that these crystals which are ordinarily called leucine are, in reality, generally urates. This view point has become more general in recent years. For the crystalline form of pure leucine obtained as a decomposition product of protein see Fig. 27, p. 85. Tyrosine crystallizes in urinary sediments in the well-known sheaf or tuft formation (Fig. 24, p. 81). For other tests on leucine and tyrosine see pages 90 and 91.

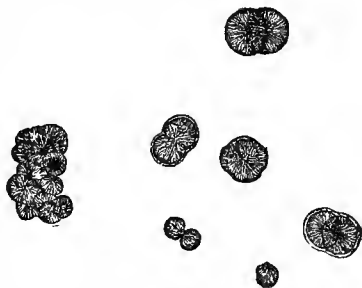


FIG. 109.—CRYSTALS OF IMPURE LEUCINE. (Ogden.)

**Hæmatoidin and Bilirubin.**—There are divergent opinions regarding the occurrence of these bodies in urinary sediment. Each of them

crystallizes in the form of tufts of small needles or in the form of small plates which are ordinarily yellowish-red in color (Fig. 42, p. 161). Because of the fact that the crystalline form of the two substances is identical many investigators claim them to be one and the same body. Other investigators claim, that while the crystalline form is the same in each case, there are certain chemical differences which may be brought out very strikingly by properly testing. For instance, it has been claimed that hæmatoidin may be differentiated from bilirubin through the fact that it gives a momentary color reaction (blue) when nitric acid is brought in contact with it, and, further, that it is not dissolved on treatment with ether or potassium hydroxide. Pathologically, typical crystals of hæmatoidin or bilirubin have been found in the urinary sediment in jaundice, acute yellow atrophy of the liver, carcinoma of the liver, cirrhosis of the liver, and in phosphorus poisoning, typhoid fever, and scarlatina.

**Magnesium Phosphate.**—Magnesium phosphate crystals occur rather infrequently in the sediment of urine which is neutral, alkaline, or *feebly* acid in reaction. It ordinarily crystallizes in elongated, highly refractive, rhombic plates which are soluble in acetic acid.

**Indigo.**—Indigo crystals are frequently found in urine which has undergone alkaline fermentation. They result from the breaking down of indoxyl-sulphates or indoxyl-glycuronates. Ordinarily indigo deposits as dark blue stellate needles or occurs as amorphous particles or broken fragments. These crystalline or amorphous forms may occur in the sediment or may form a blue film on the surface of the urine. Indigo crystals generally occur in urine which is alkaline in reaction, but they have been detected in acid urine.

**Xanthine.**—Xanthine is a constituent of normal urine but is found in the sediment in crystalline form very infrequently, and then only in pathological urine. When present in the sediment xanthine generally occurs in the form of whetstone-shaped crystals somewhat similar in form to the whetstone variety of uric acid crystal. They may be differentiated from uric acid by the great ease with which they may be brought into solution in dilute ammonia and on applying heat. Xanthine may also form urinary calculi. The clinical significance of xanthine in urinary sediment is not well understood.

**Melanin.**—Melanin is an extremely rare constituent of urinary sediments. Ordinarily in melanuria the melanin remains in solution; if it separates it is generally held in suspension as fine amorphous granules.

(b) **Organized Sediments.**

Epithelial cells.

Pus cells.

Casts.	{	Hyaline.
		Granular.
		Epithelial.
		Blood.
		Fatty.
		Waxy.
		Pus.

Cylindroids.

Erythrocytes.

Spermatozoa.

Urethral filaments.

Tissue débris.

Animal parasites.

Micro-organisms.

Fibrin.

Foreign substances due to contamination.

**Epithelial Cells.**—The detection of a certain number of these cells in urinary sediment is not, of itself, a pathological sign, since they occur in normal urine. However, in certain pathological conditions they are greatly *increased* in number, and since different areas of the urinary tract are lined with different forms of epithelial cells, it becomes necessary, when examining urinary sediments, to note not only the relative number of such cells, but at the same time to carefully observe the shape of the various individuals in order to determine, as far as possible, from what portion of the tract they have been derived. Since the different layers of the epithelial lining are composed of cells different in form from those of the associated layers, it is evident that a careful microscopical examination of these cells may tell us the particular layer which is being desquamated. It is frequently a most difficult undertaking, however, to make a clear differentiation between the various forms of epithelial cells present in the sediment. If skilfully done, such a microscopical differentiation may prove to be of very great diagnostic aid.

The principal forms of epithelial cells met with in urinary sediments are shown in Fig. 110, p. 370.

**Pus Cells.**—Pus corpuscles or leucocytes are present in extremely small numbers in normal urine. Any considerable increase in the number, however, ordinarily denotes a pathological condition, generally an acute

or chronic inflammatory condition of some portion of the urinary tract. The sudden appearance of a large amount of pus in a sediment denotes the opening of an abscess into the urinary tract. Other form elements, such as epithelial cells, casts, etc., ordinarily accompany pus corpuscles in urinary sediment and a careful examination of these associated elements is necessary in order to form a correct diagnosis as to the origin of the pus. Protein is always present in urine which contains pus.

The appearance which pus corpuscles exhibit under the microscope depends greatly upon the reaction of the urine containing them. In acid urine they generally present the appearance of round, colorless cells

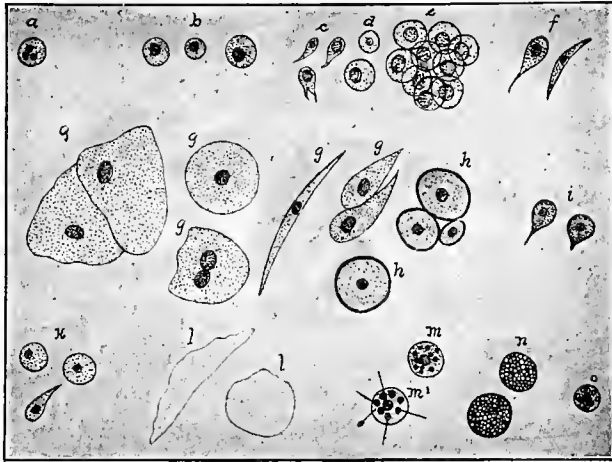


FIG. 110.—EPITHELIUM FROM DIFFERENT AREAS OF THE URINARY TRACT.

*a*, Leucocyte (for comparison); *b*, renal cells; *c*, superficial pelvic cells; *d*, deep pelvic cells; *e*, cells from calices; *f*, cells from ureter; *g, g, g, g*, squamous epithelium from the bladder; *h, h*, neck-of-bladder cells; *i*, epithelium from prostatic urethra; *k*, urethral cells; *l, l*, scaly epithelium; *m, m'*, cells from seminal passages; *n*, compound granule cells; *o*, fatty renal cell. (Ogden.)

composed of refractive, granular protoplasm, and may frequently exhibit amœboid movements, especially if the slide containing them be warmed slightly. They are nucleated (one or more nuclei), the nuclei being clearly visible only upon treating the cells with water, acetic acid, or some other suitable reagent. In urine which has a decided alkaline reaction, on the other hand, the pus corpuscles are often greatly degenerated. They may be seen as swollen, transparent cells, which exhibit no granular structure and as the process of degeneration continues the cell outline ceases to be visible, the nuclei fade, and finally only a mass of débris containing isolated nuclei and an occasional cell remains.

It is frequently rather difficult to make a differentiation between pus corpuscles and certain types of epithelial cells which are similar in form.

Such confusion may be avoided by the addition of iodine solution (I in KI), a reagent which stains the pus corpuscles a deep mahogany-brown and transmits to the epithelial cells a light yellow tint. The test proposed by Vitali often gives very satisfactory results. This simply consists in acidifying the urine (if alkaline) with acetic acid, then filtering, and treating the sediment on the filter paper with freshly prepared tincture of guaiac. The presence of pus in the sediment is indicated if a *blue* color is observed. Large numbers of pus corpuscles are present in the urinary sediment in gonorrhœa, leucorrhœa, chronic pyelitis, and in abscess of

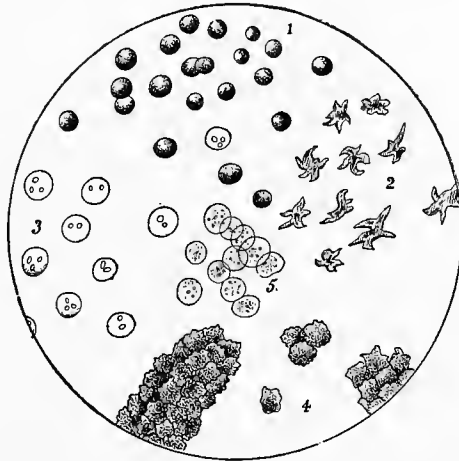


FIG. 111.—PUS CORPUSCLES. (After Ullmann.)

1, Normal; 2, showing amoeboid movements; 3, nuclei rendered distinct by acetic acid; 4, as observed in chronic pyelitis; 5, swollen by ammonium carbonate.

the kidney. In addition to the usual constituents found in leucocytes Mandel and Levene<sup>1</sup> claim that pus cells contain *glucothionic acid*.

**Casts.**—These are cylindrical formations, which originate in the uriniferous tubules and are forced out by the pressure of the urine. They vary greatly in size, but in nearly every instance they possess parallel sides and rounded ends. The finding of casts in the urine is very important because of the fact that they *generally indicate* some kidney disorder; if albumin accompanies the casts the indication is much accentuated. Casts have been classified according to their microscopical characteristics as follows: (a) Hyaline, (b) granular, (c) epithelial, (d) blood, (e) fatty, (f) waxy, (g) pus.

(a) *Hyaline Casts.*—These are composed of a basic material which is transparent, homogeneous, and very light in color (Fig. 112, p. 372). In fact, chiefly because of these physical properties, they are the most

<sup>1</sup> Mandel and Levene: *Biochemische Zeitschrift*, 4, 78, 1907.

difficult form of renal casts to detect under the microscope. Frequently such casts are impregnated with deposits of various forms, such as erythrocytes, epithelial cells, fat globules, etc., thus rendering the form of the cast more plainly visible. Staining is often resorted to in order to render the shape and character of the cast more easily determined. Ordinary iodine solution (I in KI) may be used in this connection; many of the aniline dyes are also in common use for this purpose, *e. g.*, gentian-violet, Bismarck-brown, methylene-blue, fuchsin, and eosin. Generally, but not always, albumin is present in urine containing hyaline casts. Hyaline



FIG. 112.—HYALINE CASTS.  
One cast is impregnated with four renal cells.

casts are common to all kidney disorders, but occur particularly in the earliest and recovering stages of parenchymatous nephritis and interstitial nephritis.

(*b*) *Granular Casts*.—The common hyaline material is ordinarily the basic substance of this form of cast. The granular material generally consists of albumin, epithelial cells, fat, or disintegrated erythrocytes or leucocytes, the character of the cast varying according to the nature and size of the granules (Fig. 113, p. 373, and Fig. 114, page 374). Thus we have casts of this general type classified as *finely granular* and *coarsely granular* casts. Granular casts, and in particular the finely granular types, occur in the sediment in practically every kidney disorder but are



probably especially characteristic of the sediment in inflammatory disorders.

(c) *Epithelial Casts*.—These are casts bearing upon their surface epithelial cells from the lining of the uriniferous tubules (Fig. 115, p. 374). The basic material of this form of cast may be hyaline or granular in nature. Epithelial casts are particularly abundant in the urinary sediment in *acute nephritis*.

(b) *Blood Casts*.—Casts of this type may consist of erythrocytes borne upon a hyaline or a fibrinous basis (Fig. 116, p. 374). The occurrence of such casts in the urinary sediment denotes renal hemorrhage and they are considered to be especially characteristic of *acute diffuse nephritis* and *acute congestion of the kidney*.

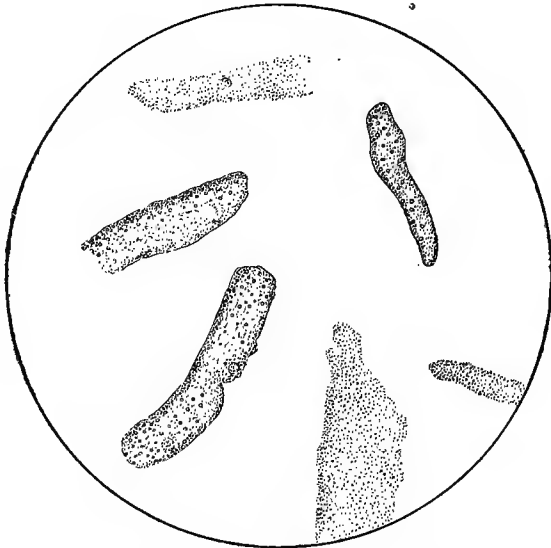


FIG. 113.—GRANULAR CASTS. (After Peyer.)

(e) *Fatty Casts*.—Fatty casts may be formed by the deposition of fat globules or crystals of fatty acid upon the surface of a hyaline or granular cast (Fig. 117, p. 375). In order to constitute a true fatty cast the deposited material must cover the greater part of the surface area of the cast. The presence of fatty casts in urinary sediment indicates fatty degeneration of the kidney; such casts are particularly characteristic of *subacute and chronic inflammation of the kidney*.

(f) *Waxy Casts*.—These casts possess a basic substance similar to that which enters into the foundation of the hyaline form of cast. In common with the hyaline type they are colorless, refractive bodies, but differ from this form of cast in being, in general, of greater length and

diameter and possessing sharper outlines and a light yellow color (Fig 118, p. 375). Such casts occur in several forms of nephritis, but do not appear to characterize any particular type of the disorder except *amyloid disease*, in which they are rather common.

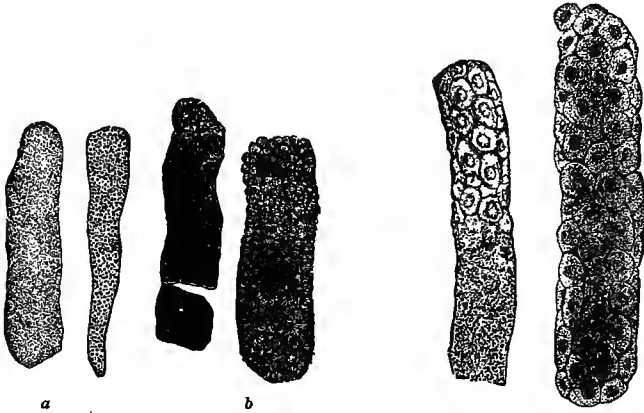


FIG. 114.—GRANULAR CASTS.

*a*, Finely granular; *b*, coarsely granular.

FIG. 115.—EPITHELIAL CASTS.

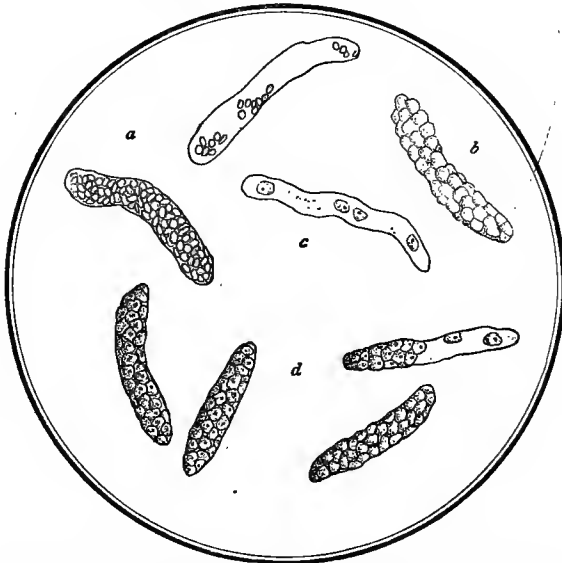


FIG. 116.—BLOOD, PUS, HYALINE AND EPITHELIAL CASTS.

*a*, Blood casts; *b*, pus cast; *c*, hyaline cast impregnated with renal cells; *d*, epithelial casts.

(*g*) *Pus Casts*.—Casts whose surface is covered with pus cells or leucocytes are termed *pus casts* (Fig. 116, above). They are frequently mistaken for epithelial casts. The differentiation between these two types is made very simple, however, by treating the cast with acetic acid

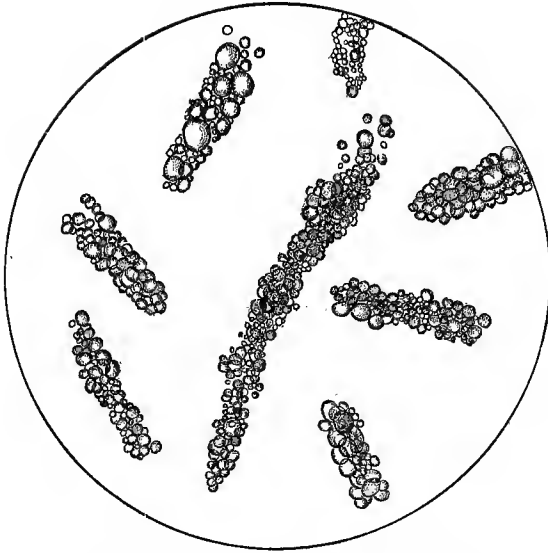


FIG. 117.—FATTY CASTS. (After Peyer.)

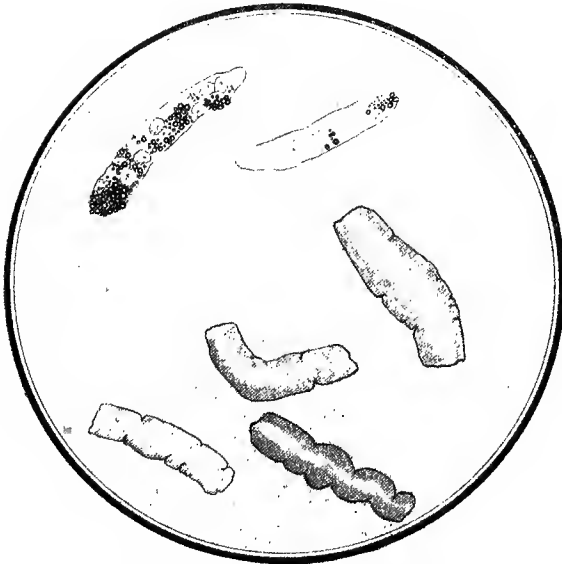


FIG 118.—FATTY AND WAXY CASTS.  
a, Fatty casts; b, waxy casts.

which causes the nuclei of the leucocytes to become plainly visible. The true pus cast is quite rare and indicates renal suppuration.

**Cylindroids.**—These formations may occur in normal or pathological urine and have no particular clinical significance. They are frequently mistaken for true casts, especially the hyaline type, but they are ordinarily *flat* in structure with a rather smaller diameter than casts, may possess forked or branching ends, and are not composed of homogenous material as are the hyaline casts. Such “false casts” may become coated with urates, in which event they appear granular in structure. The basic

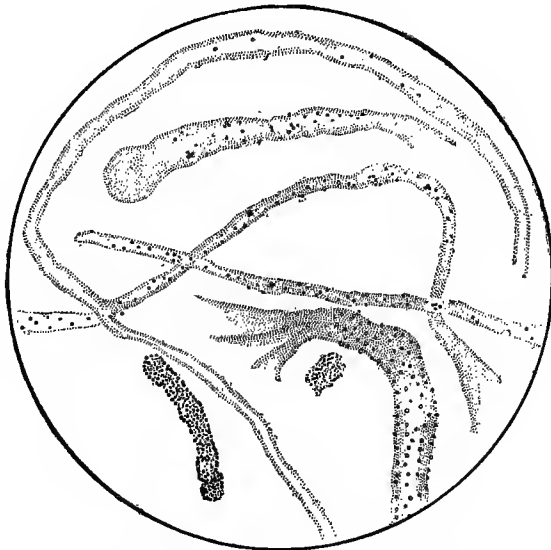


FIG. 119.—CYLINDROIDS. (After Peyer.)

substance of cylindroids is often the nucleoprotein of the urine (see Fig. 119, above).

**Erythrocytes.**—These form elements are present in the urinary sediment in various diseases. They appear as the normal biconcave, yellow erythrocyte (Plate IV, opposite page 196) or may exhibit certain modifications in form, such as the crenated type (Fig. 120, p. 377) which is often seen in concentrated urine. Under different conditions they may become swollen sufficiently to entirely erase the biconcave appearance and may even occur in the form of colorless spheres having a smaller diameter than the original disc-shaped corpuscles. Erythrocytes are found in urinary sediment in hemorrhage of the kidney or of the urinary tract, in traumatic hemorrhage, hemorrhage from congestion, and in hemorrhagic diathesis.

**Spermatozoa.**—Spermatozoa may be detected in the urinary sedi-

ment in diseases of the genital organs, as well as after coitus, nocturnal emissions, epileptic, and other convulsive attacks, and sometimes in severe febrile disorders, especially in typhoid fever. In form they consist of an

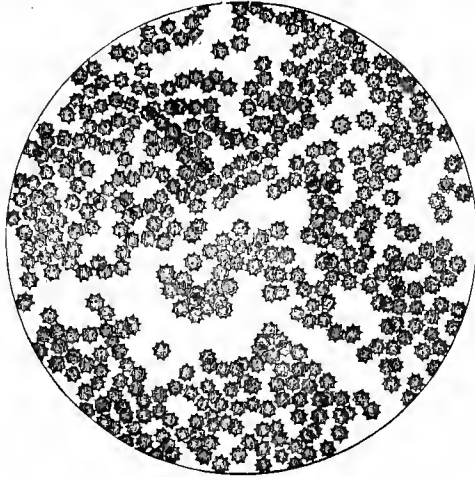


FIG. 120.—CRENATED ERYTHROCYTES.

oval body, to which is attached a long, delicate tail (Fig. 121, below). Upon examination they may show motility or may be motionless.

**Urethral Filaments.**—These are peculiar thread-like bodies which

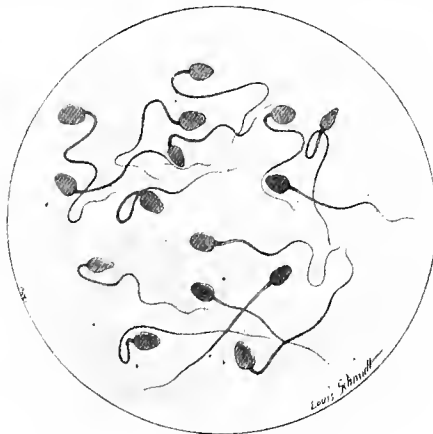


FIG. 121.—HUMAN SPERMATOZOA.

are sometimes found in urinary sediment. They may occasionally be detected in normal urine and pathologically are found in the sediment in acute and chronic gonorrhœa and in urethrorrhœa. The ground-substance of these urethral filaments is, in part at least, similar to that of the

cylindroids (see page 376). The urine first voided in the morning is best adapted for the examination for filaments. These filaments may ordinarily be removed by a pipette since they are generally macroscopic.

**Tissue Débris.**—Masses of cells or fragments of tissue are frequently found in the urinary sediment. They may be found in the sediment in tubercular affections of the kidney and urinary tract or in tumors of these organs. Ordinarily it is necessary to make a histological examination of such tissue fragments before coming to a final decision as to their origin.

**Animal Parasites.**—The cysts, hooklets, and membrane shreds of *echinococci* are sometimes found in the urinary sediments. Other animal organisms which are more rarely met with in the urine are embryos of the *Filaria sanguinis* and eggs of the *Distoma hæmatobium* and *Ascarides*. Animal parasites in general occur most frequently in the urine in tropical countries.

**Micro-organisms.**—Bacteria as well as yeasts and moulds are frequently detected in the urine. Both the pathogenic and non-pathogenic forms of bacteria may occur. The non-pathogenic forms most frequently observed are *micrococcus ureæ*, *bacillus ureæ*, and *staphylococcus ureæ liquefaciens*. Of the pathogenic forms many have been observed, e. g., *Bacterium Coli*, *typhoid bacillus*, *tubercle bacillus*, *gonococcus*, *bacillus pyocyaneus*, and *proteus vulgaris*. Yeast and moulds are most frequently met in diabetic urine.

**Fibrin.**—Following hæmaturia, fibrin clots are occasionally observed in the urinary sediment. They are generally of a semi-gelatinous consistency and of a very light color, and when examined under the microscope they are seen to be composed of bundles of highly refractive fibers which run parallel.

**Foreign Substances Due to Contamination.**—Such foreign substances as fibers of silk, linen, or wool; starch granules, hair, fat, and sputum, as well as muscle fibers, vegetable cells, and food particles are often found in the urine. Care should be taken that these foreign substances are not mistaken for any of the true sedimentary constituents already mentioned.

## CHAPTER XXI.

### URINE : CALCULI.

Urinary *calculi*, also called *concretions*, or *concrements* are solid masses of urinary sediment formed in some part of the urinary tract. They vary in shape and size according to their location, the smaller calculi, termed *sand* or *gravel*, in general arising from the kidney or the pelvic portion of the kidney, whereas the large calculi are ordinarily formed in the bladder. There are two general classes of calculi as regards composition, *i. e.*, *simple* and *compound*. The simple form is made up of but a single constituent, whereas the compound type contains two or more individual constituents. The structural plan of most calculi consists of an arrangement of concentric rings about a central nucleus, the number of rings frequently being dependent upon the number of individual constituents which enter into the structure of the calculus. In case two or more calculi unite to form a single calculus the resultant body will obviously contain as many nuclei as there were individual calculi concerned in its construction. Under certain conditions the growth of a calculus will be principally in only one direction, thus preventing the nucleus from maintaining a central location. The qualitative composition of urinary calculi is dependent, in great part, upon the reaction of the urine, *e. g.*, if the reaction of the urine is acid the calculi present will be composed, in great part at least, of substances that are capable of depositing in acid urine.

According to Ultzmann, out of 545 cases of urinary calculus, uric acid and urates formed the nucleus in about 81 per cent of the cases; earthy phosphates in about 9 per cent; calcium oxalate in about 6 per cent; cystine in something over 1 per cent, while in about 3 per cent of the cases some foreign body comprised the nucleus.

In the chemical examination of urinary calculi the most valuable data are obtained by subjecting each of the concentric layers of the calculus to a separate analysis. Material for examination may be conveniently obtained by sawing the calculus carefully through the nucleus, then separating the various layers or by scraping off from each layer (without separating the layers) enough powder to conduct the examination as outlined in the scheme (see page 381).

### Varieties of Calculus.

**Uric Acid and Urate Calculi.**—Uric acid and urates constitute the nuclei of a large proportion (81 per cent) of urinary concretions. Such stones are always colored, the tint varying from a pale yellow to a brownish-red. The surface of such calculi is generally smooth but it may be rough and uneven.

**Phosphatic Calculi.**—Ordinarily these concretions consist principally of "triple phosphate" and other phosphates of the alkaline earths, with very frequent admixtures of urates and oxalates. The surface of such calculi is generally rough but may occasionally be rather smooth. The calculi are somewhat variable in color, exhibiting gray, white, or yellow tints under different conditions. When composed of earthy phosphates the calculi are characterized by their friability.

**Calcium Oxalate Calculi.**—This is the hardest form of calculus to deal with, and is rather difficult to crush. They ordinarily occur in two general forms, *i. e.*, the small, smooth concretion which is characterized as the *hemp-seed calculus* and the medium-sized or large stone possessing an extremely uneven surface which is generally classed as a *mulberry calculus*. This roughened surface of the latter form of calculus is due, in many instances, to protruding calcium oxalate crystals of the octahedral type.

**Calcium Carbonate Calculi.**—Calcium carbonate concretions are quite common in herbivorous animals, but of exceedingly rare occurrence in man. They are generally small, white, or grayish calculi, spherical in form and possess a hard, smooth surface.

**Cystine Calculi.**—The cystine calculus is a rare variety of calculus. Ordinarily they occur as small, smooth, oval, or cylindrical concretions which are white or yellow in color and of a rather soft consistency.

**Xanthine Calculi.**—This form of calculus is somewhat more rare than the cystine type. The color may vary from white to brownish-yellow. Very often uric acid and urates are associated with xanthine in this type of calculus. Upon rubbing a xanthine calculus it has the property of assuming a wax-like appearance.

**Urostealith Calculi.**—This form of calculus is extremely rare. Such concretions are composed principally of fat and fatty acid. When moist they are soft and elastic, but when dried they become brittle. Urostealiths are generally light in color.

**Fibrin Calculi.**—Fibrin calculi are produced in the process of blood coagulation within the urinary tract. They frequently occur as nuclei of other forms of calculus. They are rarely found.



## On Heating the Powder on Platinum Foil, It

Does not burn		Does burn	
The powder when treated with HCl		With flame	Without flame
Does not effervesce		<p>Flame yellow, continuous. Odor of burnt feathers. Insoluble in alcohol and ether. Soluble in potassium hydroxide with heat. Precipitated herefrom by acetic acid and generation of hydrogen sulphide</p>	<p>The powder gives the murexide test</p>
The powder gently heated with HCl			<p>The powder when treated with KOH gives</p>
The powder when moistened with a little KOH		<p>Flame pale blue, burns a short time. Peculiar sharp odor. The powder dissolves in ammonia, and six-sided plates separate on the spontaneous evaporation of the ammonia</p>	<p>Does not give murexide test. The powder dissolves in nitric acid without effervescence. The dried yellow residue becomes orange with alkali, beautiful red on warming</p>
Abundant ammonia. The powder dissolves in acetic acid or HCl. This solution gives a crystalline precipitate with ammonia			
No ammonia or, at least, only traces of ammonia. Powder dissolves in acetic acid or HCl. This solution is precipitated by ammonia (amorphous)		<p>Flame yellow, pale, continuous. Odor of resin or shellac on burning. Powder soluble in alcohol and ether</p>	<p>Strong ammonia reaction</p>
Effervesces		<p>Effervesces</p>	<p>Uric acid.</p>
Effervesces		<p>Effervesces</p>	<p>Ammonium urate.</p>
Calcium oxalate.		<p>Calcium carbonate.</p>	<p>Xanthine.</p>
Bone-earth (magnesium and calcium phosphate).		<p>Urostealith.</p>	<p>Cystine.</p>
"Triple phosphate" (mixed with unknown amount of earthy phosphate).		<p>Fibrin.</p>	

**Cholesterol Calculi.**—An extremely rare form of calculus somewhat resembling the cystine type.

**Indigo Calculi.**—Indigo calculi are extremely rare, only two cases having been reported. One of these indigo calculi is on exhibition in the museum of Jefferson Medical College of Philadelphia.

The scheme, proposed by Heller and given on page 381, will be found of much assistance in the chemical examination of urinary calculi.

## CHAPTER XXII.

### URINE: QUANTITATIVE ANALYSIS.

#### I. Protein.

1. **Scherer's Coagulation Method.**—The content of *coagulable* protein may be accurately determined as follows: Place 50 c.c. of urine in a small beaker and raise the temperature of the fluid to about 40° C. upon a water-bath. Add dilute acetic acid, drop by drop, to the warm urine, to precipitate the protein which will separate in a flocculent form. Care should be taken not to add too much acid; ordinarily less than twenty drops is sufficient. The temperature of the water in the water-bath should now be raised to the boiling-point and maintained there for a few minutes in order to insure the complete coagulation of the protein present. Now filter the urine<sup>1</sup> through a previously *washed, dried, and weighed* filter paper, wash the precipitated protein, in turn, with hot water, 95 per cent alcohol, and with ether, and dry the paper and precipitate, to constant weight, in an air-bath at 110° C. Subtract the weight of the filter paper from the combined weight of the paper and precipitate and calculate the percentage of protein in the urine specimen.

*Calculation.*—To determine the percentage of protein present in the urine under examination, multiply the weight of the precipitate, expressed in grams, by 2.

2. **Esbach's Method.**—This method depends upon the precipitation of protein by Esbach's reagent<sup>2</sup> and the apparatus used in the estimation is Esbach's albuminometer (Fig. 122, p. 384). In making a determination fill the albuminometer to the point U with urine, then introduce the reagent until the point R is reached. Now stopper the tube, invert it slowly several times in order to insure the thorough mixing of the fluids, and stand the tube aside for 24 hours. Creatinine, resin acids, etc., are precipitated in this method, and for this and other reasons it is not as accurate as the coagulation method. It is, however, extensively used clinically. According to Sahli<sup>3</sup> the method is "accurate approxi-

<sup>1</sup> If it is desired the precipitate may be filtered off on an *unweighed* paper, and its nitrogen content determined by the Kjeldahl method (see p. 401). In order to arrive at correct figures for the protein content it is then simply necessary to multiply the total nitrogen content by 6.25 (see p. 438). Correction should be made for the nitrogen content of the filter paper used unless this factor is negligible.

<sup>2</sup> Esbach's reagent is prepared by dissolving 10 grams of picric acid and 20 grams of citric acid in 1 liter of water.

<sup>3</sup> Sahli: *Lehrbuch d. klin. Untersuchungs-Methoden*, 5th Aufl., 1909.

mately to one part per 1000," whereas Pfeiffer<sup>1</sup> claims it is not accurate for less than one-half or for more than five parts per 1000.

*Calculation.*—The graduations on the albuminometer indicate *grams of protein per liter of urine*. Thus, if the protein precipitate is level with the figure 3 of the graduated scale, this denotes that the urine examined contains 3 grams of protein to the liter. To express the amount of protein in *per cent* simply move the decimal point *one* place to the left. In the case under consideration the urine contains 0.3 per cent protein.

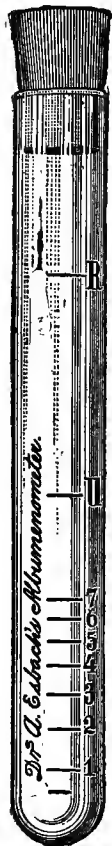


FIG. 122.—ESBACH'S ALBUMINOMETER. (Ogden.)

### 3. Kwilecki's Modification of Esbach's Method.<sup>2</sup>

—Add 10 drops of a 10 per cent solution of  $\text{FeCl}_3$  to the acid urine before introducing the Esbach's reagent. Warm the tube and contents in a water-bath at  $72^\circ \text{C}$ . for 5–6 minutes and make the reading.

## II. Dextrose.

1. **Fehling's Method.**—Place 10 c.c. of the urine under examination in a 100 c.c. volumetric flask and make the volume up to 100 c.c. with distilled water. Thoroughly mix this diluted urine by pouring it into a beaker and stirring with a glass rod, then transfer a portion of it to a burette which is properly supported in a clamp.

Now place 10 c.c. of Fehling's solution<sup>3</sup> in a small beaker, dilute it with approximately 40 c.c. of distilled water, heat to boiling, and observe whether decomposition of the Fehling's solution itself has occurred as indicated by the production of a turbidity. If such turbidity is produced the Fehling's solution is unfit for use. Clamp the burette containing the dilute urine immediately over the beaker and carefully allow from 0.5 to 1 c.c. of the diluted urine to flow into the *boiling* Fehling's solution. Bring the solution to the boiling-point after each addition of urine and continue running the urine from the burette, 0.5–1 c.c. at a time, as indicated, until the Fehling's solution is *completely reduced*, *i. e.*, until all the cupric oxide in solution has been precipitated as cuprous oxide. This point will be indicated by the *absolute disappearance of all blue color*. When this end-point is reached

<sup>1</sup>Pfeiffer: *Berl. klin. Woch.*, 49, 114, 1912.

<sup>2</sup>Kwilecki: *Munch. Med. Woch.*, 56, p. 1330.

<sup>3</sup>Directions for the preparation of Fehling's solution are given in a note at the bottom of page 32.

note the number of cubic centimeters of diluted urine used in the process and calculate the percentage of dextrose present, in the sample of urine analyzed, according to the method given below.

This is a very satisfactory method, the main objection to its use being the uncertainty attending the determination of the end-reaction, *i. e.*, the difficulty with which the exact point where the blue color *finally disappears* is noted. Several means of accurately fixing this point have been suggested, but they are practically all open to objection. As good a "check" as any, perhaps is to filter a few drops of the solution, through a double paper, after the blue color has *apparently* disappeared, acidify the filtrate with acetic acid and add potassium ferrocyanide. If the copper of the Fehling's solution has been completely reduced, there will be no color reaction, whereas the production of a brown color indicates the presence of *unreduced* copper. Harrison has recently suggested the following procedure to determine the exact end-point: To about 1 c.c. of a starch iodide solution<sup>1</sup> in a test-tube add 2-3 drops of acetic acid and introduce into the acidified mixture 1-2 drops of the solution to be tested. *Unreduced copper* will be indicated by the production of a *purplish-red* or *blue* color due to the liberation of iodine.

It is ordinarily customary to make at least three determinations by Fehling's method before coming to a final conclusion regarding the sugar content of the urine under examination.

*Calculation.*—Ten c.c. of Fehling's solution is completely reduced by 0.05 gram of dextrose.<sup>2</sup> If  $y$  represents the number of cubic centimeters of *undiluted* urine (obtained by dividing the burette reading by 10) necessary to reduce the 10 c.c. of Fehling's solution, we have the following proportion:

$$y : 0.05 :: 100 : x \text{ (percentage of dextrose).}$$

2. **Benedict's Method No. 1.**—To 30 c.c. of Benedict's solution<sup>3</sup> in a small beaker add from 2.5 grams to 5 grams of anhydrous sodium car-

<sup>1</sup> The starch-iodide solution may be prepared as follows: Mix 0.1 gram of starch with *cold* water in a mortar and pour the suspended starch granules into 75-100 c.c. of *boiling* water, stirring continuously. Cool the starch paste, add 20-25 grams of potassium iodide and dilute the mixture to 250 c.c. This solution deteriorates upon standing, and therefore must be freshly prepared as needed.

<sup>2</sup> The values for certain other sugars are as follows:

Lactose.....	0.0676 gram.
Maltose.....	0.074 gram.
Invert sugar.....	0.0475 gram.

<sup>3</sup> Benedict's solution used in the quantitative determination of sugar consists of *three* separate solutions. The *copper sulphate solution* and the *alkaline tartrate solution* are the same as those already described in connection with Benedict's qualitative test, see p. 33. The third solution is made up as follows:

*Potassium ferro-thiocyanate solution* = 15 grams of potassium ferrocyanide, 62.5 grams of potassium thiocyanate and 50 grams of anhydrous sodium carbonate dissolved in water and made up to 500 c.c.

These *three* solutions should be preserved *separately* in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

bonate<sup>1</sup> and heat the mixture to boiling over a wire gauze until the carbonate has been brought into solution.

Place the urine under examination in a burette and run it into the hot Benedict solution rather rapidly<sup>2</sup> until the formation of a heavy *chalk-white precipitate* is noted and the blue color of the solution lessens perceptibly in its intensity. From this point in the determination from 2 to 10 drops<sup>3</sup> of the urine should be run into the boiling Benedict solution at one time, boiling the solution vigorously for about 15 seconds after each addition. Complete reduction of the copper is indicated here as in Fehling's original method, by the *complete disappearance of all blue color*. The end-point here, however, is very sharply defined, contrary to the conditions in the older method.

To prevent the annoying bumping which often interferes with the titration, a medium-sized piece of washed absorbent cotton<sup>4</sup> may be introduced into the solution. This cotton may be stirred about through the solution as the titration proceeds and the bumping thus eliminated.

*Calculation.*—Thirty cubic centimeters of Benedict's solution is completely reduced by 0.073 gram of *dextrose*. If  $y$  represents the number of cubic centimeters of urine necessary to reduce the 30 c.c. of the solution we have the following proportion:

$$y : 0.073 :: 100 : x \text{ (percentage of dextrose).}$$

**Benedict's Method No. 2.**<sup>5</sup>—"The urine, 10 c.c. of which should be diluted with water to 100 c.c. (unless the sugar content is believed to be low), is poured into a 50 c.c. burette up to the zero mark. Twenty-five cubic centimeters of the reagent<sup>6</sup> are measured with a pipette into a porcelain evaporation dish (25–30 cm. in diameter), 10 to 20 grams of crystallized sodium carbonate (or one-half the weight of the anhydrous

<sup>1</sup> The amount added depends upon the dilution to which the solution is to be subjected in titration. For this reason the maximum amount of sodium carbonate should be added when titrating urines containing a very low percentage of sugar.

<sup>2</sup> Not rapidly enough, however, to interfere in any marked degree with the continuous vigorous boiling of the solution.

<sup>3</sup> The exact amount to run in depends upon the intensity of the remaining blue color, as well as upon the sugar content of the urine. The 10 drops should be added at one time only when urines containing a very low percentage of sugar are under examination.

<sup>4</sup> Glass wool may be substituted if desired.

<sup>5</sup> Benedict: *Jour. Am. Med. Ass'n.*, 57, 1193, 1911.

<sup>6</sup> Copper sulphate (crystallized).....	18.0 grams.
Sodium carbonate (crystallized, one-half the weight of the anhydrous salt may be used).....	200.0 grams.
Sodium or potassium citrate.....	200.0 grams.
Potassium thiocyanate.....	125.0 grams.
Potassium ferrocyanide (5 per cent solution).....	5.0 c.c.
Distilled water to make a total volume of.....	1000.0 c.c.

With the aid of heat dissolve the carbonate, citrate and thiocyanate in enough water to make about 800 c.c. of the mixture and filter if necessary. Dissolve the copper sulphate separately in about 100 c.c. of water and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanide solution, cool and dilute to exactly 1 liter. Of the various constituents, the copper salt only need be weighed with exactness. Twenty-five cubic centimeters of the reagent are reduced by 50 mg. of glucose.

salt) are added, together with a small quantity of powdered pumice stone or talcum, and the mixture heated to boiling over a free flame until the carbonate has entirely dissolved. The diluted urine is now run in from the burette, rather rapidly until a chalk-white precipitate forms, and the blue color of the mixture begins to lessen perceptibly, after which the solution from the burette must be run in a few drops at a time, until the disappearance of the last trace of blue color, which marks the end-point. The solution must be kept vigorously boiling throughout the entire titration. If the mixture becomes too concentrated during the process, water may be added from time to time to replace the volume lost by evaporation. The calculation of the percentage of sugar in the original sample of urine is very simple. The 25 c.c. of copper solution are reduced by exactly 50 mg. of glucose. Therefore the volume run out of the burette to effect the reduction contained 50 mg. of the sugar. When the urine is diluted 1:10, as in the usual titration of diabetic urines, the formula for calculating the per cent of the sugar is the following:

$$\frac{0.050}{X} \times 1000 = \text{per cent in original sample, wherein } X \text{ is the number of cubic centimeters of the diluted urine required to reduce 25 c.c. of the copper solution.}''$$

“In the use of this method chloroform must not be present during the titration. If used as a preservative in the urine it may be removed by boiling a sample for a few minutes, and then diluting to its original volume.”

“Like the reagent for qualitative employment, the one for quantitative work will keep indefinitely after its preparation. As regards the accuracy of the method, it may be stated that repeated determinations, and comparisons with results by the polariscope and by Allihn’s gravimetric process have shown the method to be probably more exact than any other titration method available for sugar work.”

3. **Purdy’s Method.**—Purdy’s solution<sup>1</sup> is a modification of Fehling’s solution and is said to possess greater stability than the latter. One of the most satisfactory points about the method as suggested by Purdy is the ease with which the exact end-reaction may be determined. In determining the percentage of dextrose by this method proceed as follows:

<sup>1</sup> Purdy’s solution has the following composition:

Copper sulphate.....	4.752 grams.
Potassium hydroxide.....	23.5 grams.
Ammonia (U. S. P., sp. gr. 0.9).....	350.0 c.c.
Glycerol.....	38.0 c.c.
Distilled water, to make total volume 1 liter.	

In preparing the solution bring the copper sulphate and potassium hydroxide into solution in separate vessels, mix the two solutions, cool the mixture, and add the ammonia and glycerol. After this has been done the total volume should be made up to 1 liter with distilled water.

Thirty-five cubic centimeters of Purdy’s solution is exactly reduced by 0.02 gram of dextrose.

Place 35 c.c. of Purdy's solution in a 200 c.c. Erlenmeyer flask and dilute the fluid with approximately two volumes of distilled water. Fit a cork, provided with two perforations, to the neck of the flask and through one perforation introduce the tip of a burette and through the second perforation introduce a tube bent at right angles in such a manner as to allow the steam to escape and keep the fumes of ammonia away from the face of the operator as completely as possible.<sup>1</sup> Now bring the solution to the boiling-point and add the urine, drop by drop, until the intensity of the *blue color begins to diminish*. When this point is reached add the urine somewhat *more slowly* until the blue color is entirely dissipated and an *absolutely decolorized* solution remains. Take the burette reading and calculate the percentage of dextrose in the urine examined according to the method given below.

Care should be taken not to boil the solution for too long a period, since, under these conditions, sufficient ammonia might be lost to allow the cuprous hydroxide to precipitate.

Some investigators consider it to be advisable to dilute the urine before applying the above manipulation, but ordinarily this is not necessary unless the urine has a high content of dextrose (5 per cent or over). In this event the urine may be diluted with 2-3 volumes of water and the proper correction made in the calculation.

*Calculation.*—Thirty-five c.c. of Purdy's solution is completely reduced by 0.02 gram of dextrose. If  $y$  represents the number of cubic centimeters of *undiluted* urine necessary to reduce 35 c.c. of Purdy's solution, we have the following proportion:

$$y : 0.02 :: 100 : x \text{ (percentage of dextrose).}$$

4. **Fermentation Method.**—This method consists in the measurement of the volume of carbon dioxide evolved when the dextrose of the urine undergoes fermentation with yeast. None of the various methods whose manipulation is based upon this principle is *absolutely* accurate. The method in which Einhorn's saccharometer (Fig. 3, page 36) is the apparatus employed is perhaps as satisfactory as any for clinical purposes. The procedure is as follows: Place about 15 c.c. of urine in a mortar, add about 1 gram of yeast ( $1/16$  of the ordinary cake of compressed yeast) and carefully crush the latter by means of a pestle. Transfer the mixture to the saccharometer, being careful to note that the graduated tube is *completely* filled and that no air bubbles gather at the

<sup>1</sup> This side tube may also be equipped with a simple air-valve, thus insuring the exclusion of air and thereby contributing to the accuracy of the determination, inasmuch as the cuprous salts would be reoxidized upon coming in contact with the air. If one is careful to maintain the solution continuously at the boiling-point throughout the entire process, however, there is no opportunity for air to enter and therefore no need of an air-valve.



top. Allow the apparatus to stand in a warm place (30° C.) for 12 hours and observe the percentage of dextrose as indicated by the graduated scale of the instrument. Both the percentage of dextrose and the number of cubic centimeters of carbon dioxide are indicated by the graduations on the side of the saccharometer tube.

The availability of the fermentation procedure as a quantitative aid has been appreciably lowered through the important findings of Neuberg and Associates<sup>1</sup> recently reported. They show that yeast has the property of *splitting off carbon dioxide from the carboxyl group of amino and other aliphatic acids*. The active agent in this "sugar-free fermentation" is an enzyme called *carboxylase*. Inasmuch as amino acids are always present in the urine, the error from this source is apparent.

**5. Polariscopic Examination.**—Before subjecting urine to a polariscopic examination the slightly acid fluid should be decolorized as thoroughly as possible by the addition of a little lead acetate. The urine should be well stirred and then filtered through a filter paper which has not been previously moistened. In this way a perfectly *clear* and almost colorless liquid is obtained.

In determining dextrose by means of the polariscope it should be borne in mind that this carbohydrate is often accompanied by other optically active substances, such as proteins, lævulose,  $\beta$ -oxybutyric acid, and conjugate glycuronates which may introduce an error into the polariscopic reading; the method is, however, sufficiently accurate for practical purposes.

For directions as to the manipulation of the polariscope see page 36.

### III. Uric Acid.

**1. Folin-Shaffer Method.**—Introduce 100 c.c.<sup>2</sup> of urine into an Erlenmeyer flask, add 25 c.c. of the Folin-Shaffer reagent<sup>3</sup> and after shaking the flask to thoroughly mix the fluids allow the mixture to stand,<sup>4</sup> with or without further stirring, until the precipitate has settled (5–10 minutes). Filter, transfer 100 c.c. of the filtrate to a 200 c.c. Erlenmeyer flask, add 5 c.c. of concentrated ammonium hydroxide and allow the mixture to stand for 24 hours. Transfer the precipitated ammonium urate quantitatively to a filter paper,<sup>5</sup> using 10 per cent

<sup>1</sup> Neuberg and Associates: *Biochemische Zeitschrift*, 31, 170; 36 (60, 68, and 76), 1911.

<sup>2</sup> It is preferable to use more than 100 c.c. of urine if the fluid has a specific gravity less than 1.020.

<sup>3</sup> The Folin-Shaffer reagent consists of 500 grams of ammonium sulphate, 5 grams of uranium acetate and 60 c.c. of 10 per cent acetic acid in 650 c.c. of distilled water.

<sup>4</sup> The mixture should not be allowed to stand for too long a time at this point, since uric acid may be lost through precipitation.

<sup>5</sup> The Schleicher and Schüll *hardened* papers or the Baker and Adamson *washed, ashless* variety are very satisfactory for this purpose.

ammonium sulphate to remove the final traces of the urate from the flask. Wash the precipitate *approximately* free from chlorides by means of 10 per cent ammonium sulphate solution,<sup>1</sup> remove the paper from the funnel, open it, and by means of *hot* water rinse the precipitate back through the funnel into the flask in which the urate was originally precipitated. The volume of fluid at this point should be about 100 c.c. Cool the solution to room temperature, add 15 c.c. of concentrated sulphuric acid and titrate at once with N/20 potassium permanganate,  $K_2Mn_2O_8$ , solution. The first tinge of pink color which extends throughout the fluid after the addition of *two drops* of the permanganate solution, while stirring with a glass rod, should be taken as the end-reaction. Take the burette reading and compute the percentage of uric acid present in the urine under examination.

*Calculation.*—Each cubic centimeter of N/20 potassium permanganate solution is equivalent to 3.75 milligrams (0.00375 gram) of uric acid. The 100 c.c. from which the ammonium urate was precipitated is equivalent to only four-fifths of the 100 c.c. of urine originally taken, therefore we must take five-fourths of the burette reading in order to ascertain the number of cubic centimeters of the permanganate solution required to titrate 100 c.c. of the *original urine* to the correct end-point. If  $y$  represents the number of cubic centimeters of the permanganate solution required, we may make the following calculation:

$$y \times 0.00375 = \text{weight of uric acid in 100 c.c. of urine.}$$

Because of the solubility of the ammonium urate a correction of 3 milligrams should be added to the final result.

Calculate the quantity of uric acid in the twenty-four-hour urine specimen.

2. **Heintz Method.**—This is a very simple method and was the first one in general use for the quantitative determination of uric acid. It is believed to be somewhat less accurate than the method just described. The procedure is as follows: Place 100 c.c. of filtered urine in a beaker, add 5 c.c. of concentrated hydrochloric acid, stir the fluid thoroughly, and stand it away in a cool place for 24 hours. Filter off the uric acid crystals upon a washed, dried and *weighed* filter paper and wash them with *cold* distilled water, a few cubic centimeters at a time until the chlorides are removed. Now wash, in turn, with alcohol and with ether and finally dry the paper and crystals to constant weight at 110° C. In the process of washing the uric acid free from chlorides an error is introduced, since every cubic centimeter of water so used dissolves 0.00004 gram of uric acid.<sup>2</sup> For this reason a correction is necessary.

<sup>1</sup> This washing may be conveniently done by *decantation* if desired, thus retaining the major portion of the precipitate in the flask.

<sup>2</sup> His and Paul: *Zeit. physiol. Chem.* 21, 1, 1000.

It has been suggested that the pigment of the crystals is equivalent in weight to the amount of uric acid dissolved by the first 30 c.c. of water, and this factor should be taken into account in the computation of the percentage of uric acid.

*Calculation.*—Since 100 c.c. of urine was used the *corrected* weight of the uric acid crystals, in grams, will express the *percentage* of uric acid present.

3. **Krüger and Schmidt's Method.**—This method serves for the detection of both uric acid and the purine bases. The principle involved is the precipitation of both the uric acid and the purine bases in combination with copper oxide and the subsequent decomposition of this precipitate by means of sodium sulphide. The uric acid is then precipitated by means of hydrochloric acid and the purine bases are separated from the filtrate in the form of their copper or silver compounds. The nitrogen content of the precipitates of uric acid and purine bases is then determined by means of the Kjeldahl method (see p. 401) and the corresponding values for uric acid and purine bases calculated. The method is as follows: To 400 c.c. of albumin-free urine<sup>1</sup> in a liter flask,<sup>2</sup> add 24 grams of sodium acetate, 40 c.c. of a solution of sodium bisulphite<sup>3</sup> and heat the mixture to boiling. Add 40–80 c.c.<sup>4</sup> of a 10 per cent solution of copper sulphate and maintain the temperature of the mixture at the boiling-point for at least three minutes. Filter off the flocculent precipitate, wash it with hot water until the wash water is colorless, and return the washed precipitate to the flask by puncturing the tip of the filter paper and washing the precipitate through by means of hot water. Add water until the volume in the flask is approximately 200 c.c., heat the mixture to boiling, and decompose the precipitate of copper oxide by the addition of 30 c.c. of sodium sulphide solution.<sup>5</sup> After decomposition is complete, the mixture should be acidified with acetic acid and heated to boiling until the separating sulphur collects in a mass. Filter the hot fluid by means of a filter pump, wash with hot water, add 10 c.c. of 10 per cent hydrochloric acid and evaporate the filtrate in a porcelain dish until the total volume has been reduced to about ten cubic centimeters. Permit this residue to stand

<sup>1</sup> If albumin is present, the urine should be heated to boiling, acidified with acetic acid and filtered.

<sup>2</sup> The total volume of urine for the twenty-four hours should be sufficiently diluted with water to make the total volume of the solution 1600–2000 c.c.

<sup>3</sup> A solution containing 50 grams of Kahlbaum's commercial sodium bisulphite in 100 c.c. of water.

<sup>4</sup> The exact amount depending upon the content of the purine bases.

<sup>5</sup> This is made by saturating a 1 per cent solution of sodium hydroxide with hydrogen sulphide gas and adding an equal volume of 1 per cent sodium hydroxide.

Ordinarily the addition of 30 c.c. of this solution is sufficient, but the presence of an excess of sulphide should be *proven* by adding a drop of lead acetate to a drop of the solution. Under these conditions a dark brown color will show the presence of an excess of sodium sulphide.

about two hours to allow for the separation of the uric acid, leaving the purine bases in solution. Filter off the precipitate of uric acid, using a small filter paper, and wash the uric acid, with water made acid with sulphuric acid, until the total volume of the original filtrate and the wash water aggregates 75 c.c. Determine the nitrogen content of the precipitate by means of the Kjeldahl method (see p. 401) and calculate the uric acid equivalent.

*Calculation.*—In calculating the uric acid value from the total nitrogen simply multiply the latter by *three* and add 0.0035 to the product as a correction for the uric acid remaining in solution in the 75 c.c.

#### IV. Urea.

1. **Knop-Hüfner Hypobromite Method (using Marshall's Urea Apparatus).**—Place the thumb over the side opening of the bulb-tube of the apparatus (Fig. 123) and carefully fill the tube with sodium hypobromite solution.<sup>1</sup>

Close the opening in the end of the tube with a rubber stopper, incline the tube to allow air-bubbles to escape, and finally invert the tube and fix the stoppered end in the saucer-shaped vessel. By means of the graduated pipette *rapidly* introduce 1 c.c. of urine<sup>2</sup> into the hypobromite solution through the side opening of the bulb-tube. Withdraw the pipette immediately after the urine has been introduced. When the decomposition of the urea is completed (10–20 minutes) gently tap the bulb-tube with the finger in order to dislodge any gas bubbles which may have collected on the inner surface of the glass. The atmospheric pressure should now be equalized by attaching the funnel-tube to the bulb-tube at the side opening and introducing hypobromite solution into it until the columns of liquid in

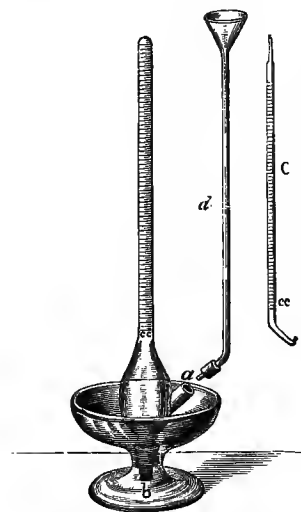


FIG. 123.—MARSHALL'S UREA APPARATUS. (Tyson.)  
a, Bulb measuring tube; b, saucer-shaped vessel; c, graduated pipette; d, funnel-tube.

<sup>1</sup> The ingredients of the sodium hypobromite solution should be prepared in the form of *two* separate solutions. When needed for use mix one volume of solution a, one volume of solution b, and 3 volumes of water.

(a) Dissolve 125 grams of sodium bromide in water, add 125 grams of bromine and make the total volume of the solution 1 liter.

(b) A solution of sodium hydroxide having a specific gravity of 1.250. This is approximately a 22.5 per cent solution.

Preserve both solutions in rubber-stoppered bottles.

<sup>2</sup> Ordinarily 1 c.c. of urine is sufficient; more may be used, however, if its content of urea is *very low*.

the two tubes are uniform in height. The graduated scale of the bulb-tube should now be read in order to determine the number of cubic centimeters of nitrogen gas evolved. By means of the appended formula the *weight* of the urea present in the urine under examination may be computed.

*Calculation.*<sup>1</sup>—By properly substituting in the following formula the *weight* of urea, in grams, contained in the volume of urine decomposed (1 c.c. or more) may readily be determined:

$$w = \frac{v(p - T)}{354.5 \times 760(1 + 0.003665t)}$$

$w$  = weight of urea, in grams.

$v$  = observed volume of nitrogen expressed in cubic centimeters.

$p$  = barometric pressure expressed in mm. of mercury.

$T$  = tension of aqueous vapor<sup>2</sup> for temperature  $t$ .

$t$  = temperature (centigrade).

If we wish to calculate the *percentage* of urea we may do so by means of the following proportion in which  $y$  represents the volume of urine used and  $w$  denotes the weight of the urea contained in the volume  $y$ :

$$y : w :: x : (\text{percentage of urea}).$$

Sodium hypobromite solution may also be employed for the determination of urea in the apparatus devised by Hufner which is pictured in Fig. 124, page 394.

2. **Knop-Hufner Hypobromite Method (Using the Doremus-Hinds Ureometer).**—In common with the method already described, this method depends upon the measurement of the volume of nitrogen gas liberated when the urea of the urine is decomposed by means of sodium hypobromite solution. The Doremus-Hinds ureometer (Fig. 125, p. 395), is one of the simplest and cheapest forms of apparatus in general use for the determination of urea by the hypobromite process. In using this apparatus proceed as follows: Fill the side tube B and the lumen of the stopcock C with the urine under examination. Carefully wash out tube A with water and introduce into it sodium hypobromite solution,<sup>3</sup> being

<sup>1</sup> 0.003665 = coefficient of expansion of gases for 1° C. 354.5 = number of c.c. of nitrogen gas evolved from 1 gram of urea.

<sup>2</sup> The values of T for the temperatures ordinarily met with are given in the following table:

Temp.	Tension in mm.	Temp.	Tension in mm.
15° C.....	12.677	21° C.....	18.505
16° C.....	13.519	22° C.....	19.675
17° C.....	14.009	23° C.....	20.909
18° C.....	15.351	24° C.....	22.211
19° C.....	16.345	25° C.....	23.582
20° C.....	17.396		

<sup>3</sup> For directions as to the preparation of this solution see page 392.

careful to fill the bulb sufficiently full to prevent the entrance of air into the graduated portion. Now allow 1 c.c. of urine<sup>1</sup> to flow from tube B into tube A and after the evolution of gas bubbles has ceased (10–20 minutes) take the reading of the graduated scale on tube A.

In common with all other methods which are based upon the decomposition of urea by means of hypobromite solution, this method is not absolutely correct. It is, however, sufficiently accurate for ordinary clinical purposes.

*Calculation.*—Observe the reading on the graduated scale of tube A. This tube is so graduated as to represent the weight of urea, in grams, per cubic centimeter of urine. If we wish to compute the *percentage* of urea present this may be done very readily by simply moving the decimal point *two places to the right*; e. g., if the reading is 0.02 gram the urine contains 2 per cent of urea.

3. **Folin's Method.**—This is one of the most accurate methods yet devised for the determination of urea in the urine. It has, however, been replaced to a great extent by the very recent modification of Folin and Pettibone (see p. 397). The procedure is as follows: Place 5 c.c. of urine in a 200 c.c. Erlenmeyer flask and add to it 5 c.c. of concentrated hydrochloric acid, 20 grams of crystallized magnesium chloride, a piece of paraffin the size of a hazel nut, and

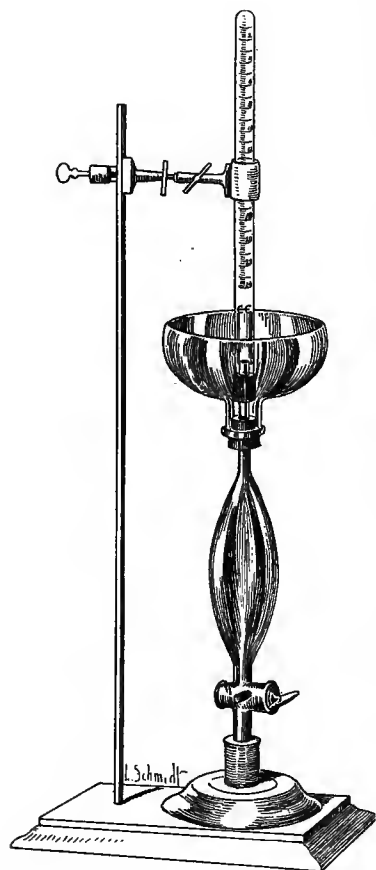


FIG. 124.—HÜFNER'S UREA APPARATUS.

2–3 drops of a 1 per cent aqueous solution of "alizerin red." Insert a Folin safety tube (Fig. 126, p. 396) into the neck of the flask and boil the mixture until each drop of reflow from the safety tube produces a very perceptible bump; the heat is then reduced somewhat and continued one and one-half hours. The contents of the flask must not remain alkaline, and to obviate this, at the first appearance of a reddish tinge in the contents of the flask *a few drops* of the acid distillate are shaken back into

<sup>1</sup> If the content of urea in the urine under examination is large, the urine may be diluted with water before determining the urea. If this is done it must of course be taken into consideration in computing the content of urea.

the flask. At the end of 1 1/2 hours the contents of the vessel are transferred to a 1-liter flask with about 700 c.c. of distilled water, about 20 c.c. of 10 per cent potassium hydroxide or sodium hydroxide solution is added and the mixture distilled into a known volume of N/10 sulphuric acid until the contents of the flask are nearly dry or until the distillate fails to give an alkaline reaction to litmus, showing the absence of ammonia. The time devoted to this process is ordinarily about an hour. Boil the distillate a few moments to free it from CO<sub>2</sub>, then cool and titrate the mixture with N/10 sodium hydroxide, using "alizarin red" as indicator.

A "check" experiment should always be made to determine the original ammonia content of the urine and of the magnesium chloride, if it is not absolutely pure, which of course should be subtracted from the total amount of ammonia as determined by the above process.

The Folin method is extremely accurate under all conditions *except when the urine contains sugar*. When this is the case the carbohydrate and the urea unite, upon being heated, and form a very stable combination. For this reason the Folin method is not suitable for use in the examination of such urines. Under such conditions the combination Mörner-Sjöqvist-Folin method which is given below or the method of Folin and Denis (p. 398) may be used.

4. **Mörner-Sjöqvist-Folin Method.**—As has already been stated in the last experiment, this method excels the Folin method in accuracy *only* in the determination of urea in the presence of carbohydrate bodies. Briefly, the procedure is as follows:<sup>1</sup> Bring the major portion of 1.5 gram of powdered barium hydroxide into solution in 5 c.c. of urine in a small flask, and treat the mixture with 100 c.c. of an alcohol-ether solution, consisting of two volumes of 97 per cent alcohol and one volume of ether. Stopper the flask and allow it to stand 12–24 hours. Filter off the precipitate, wash it with the alcohol-ether mixture and remove the alcohol

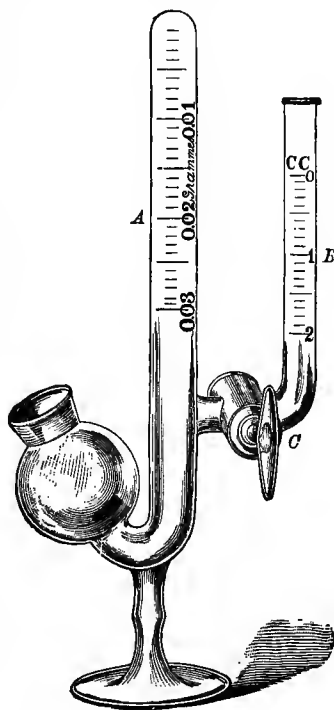


FIG. 125.—DOREMUS-HINDS  
UREOMETER.

<sup>1</sup> The original description of the method may be found in an article by Mörner: *Skandinavisches Archiv für Physiologie*, 14, 297, 1903.

and ether from the filtrate by distillation, being careful to keep the temperature of the mixture below  $50^{\circ}\text{C}$ .<sup>1</sup> Treat the remaining fluid (about 25 c.c.) with 2 c.c. of hydrochloric acid (sp. gr. 1.124), transfer it carefully to a 200 c.c. flask, and evaporate the mixture to dryness on a water-bath. Now add 20 grams of crystallized magnesium chloride and 2 c.c. of concentrated hydrochloric acid to the residue, and after fitting the flask with

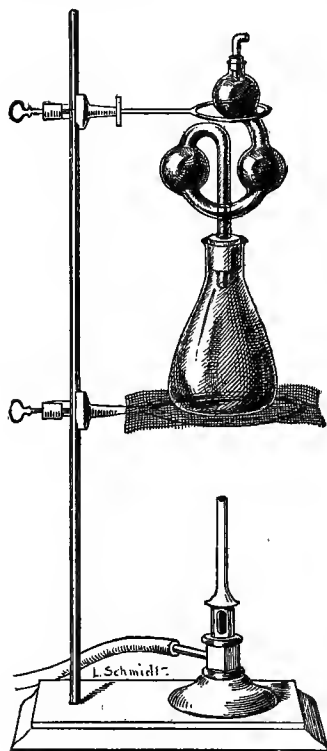


FIG. 126.—FOLIN'S UREA APPARATUS.

a return cooler boil the mixture on a wire gauze over a small flame for two hours. Cool the solution, dilute to 750 c.c. or 1000 c.c. with water, render the mixture alkaline with potassium hydroxide or sodium hydroxide, distil off the ammonia and collect it in an acid solution of known strength. Boil the distillate to remove carbon dioxide, cool and titrate with an alkali of known strength. In this method, as well as in Folin's method (see p. 394), correction must be made for the ammonia originally present in the urine and in the magnesium chloride.

5. **Benedict's Method.**<sup>2</sup>—Five cubic centimeters of urine are introduced into a rather wide Jena glass test-tube, about 3 grams of potassium bisulphate and 1–2 grams of zinc sulphate<sup>3</sup> added, a small quantity of powdered pumice and a bit of paraffin are introduced and the mixture boiled almost to dryness either over a free flame or by immersion in a sulphuric acid bath at about  $130^{\circ}$ . The tubes are then weighted (a screw clamp is convenient) and immersed for three-fourths of their length in a bath of sulphuric acid at a temperature of  $162\text{--}165^{\circ}$  (not lower) for one hour.

The contents of the tube are then washed into an 800 c.c. Kjeldahl distillation flask, diluted to about 400 c.c. with water, made alkaline by the addition of 15–20 c.c. of 10 per cent KOH (or 25 c.c. 15 per cent  $\text{Na}_2\text{CO}_3$ ) and distilled as usual in the Kjeldahl method (page 401). The value obtained must be corrected for ammonia.

<sup>1</sup> There is some decomposition of urea at  $60^{\circ}\text{C}$ .

<sup>2</sup> Benedict: *Jour. Biol. Chem.*, 8, 405, 1911.

<sup>3</sup> An excess of zinc salt is to be avoided as too large quantity tends to cause slight frothing during the final distillation.



Welker<sup>1</sup> has suggested an electrical bath for use in the first part of this method.

6. **Method of Folin and Pettibone, No. 1.**<sup>2</sup>—By means of an Ostwald pipette (see page 403) introduce 1 c.c. of urine into a Jena test-tube (20–25 mm. by 200 mm.). Add three good-sized drops of pure phosphoric acid, one drop of indicator (alizarin) and a few grains of talcum powder and concentrate the mixture to one-half its volume by boiling over a free flame for 2–3 minutes. At the end of this time heat the test-tube in a bath of sulphuric acid, oil, or paraffin, for fifteen minutes at a temperature of 175–180° C.<sup>3</sup> By this means the urea is decomposed with the formation of ammonium phosphate. Dissolve the contents of the tube in water (1–2 c.c.) with the aid of heat, make alkaline with potassium hydroxide<sup>4</sup> (0.5–1 c.c. of a 50 per cent solution) and remove the liberated ammonia by means of a strong air current (see page 404). This process requires approximately ten minutes. The ammonia may be collected in 25 c.c. of *N*/50 hydrochloric acid and the excess acid titrated with *N*/100 sodium hydroxide using alizarin as indicator.

In calculating the urea value a correction must be made for the ammonia content of the urine.

With the bath previously heated to the proper temperature the above method may be completed in about one-half hour.

7. **Method of Folin and Pettibone, No. 2.**<sup>5</sup>—Dilute the urine so that 1 c.c. contains 0.75–1.5 mg. of urea nitrogen. Generally dilutions of 1:20 or 1:10, depending on the concentration, are satisfactory. By means of an Ostwald pipette (see page 403) introduce 1 c.c. of the diluted urine into a large dry Jena test-tube (20–25 mm. by 200 mm.) which already contains 7 grams of dry *ammonia-free* potassium acetate<sup>6</sup> (*free from lumps*), 1 c.c. of 50 per cent acetic acid, a small sand pebble or a little powdered zinc (not zinc *dust*) to prevent bumping during boiling, and a temperature indicator.<sup>7</sup>

<sup>1</sup> Welker: *Biochemical Bulletin*, 1, 439, 1912.

<sup>2</sup> Folin and Pettibone: *Jour. Biol. Chem.*, 11, 512, 1912.

<sup>3</sup> The bath should be at this temperature when the tubes are introduced. Welker's electric bath may be used in this connection. (See *Biochemical Bulletin*, 1, 439, 1912).

<sup>4</sup> Potassium hydroxide is preferred to sodium hydroxide because of the greater solubility of potassium phosphate.

<sup>5</sup> Folin and Pettibone: *Jour. Biol. Chem.*, 11, 513, 1912.

<sup>6</sup> A satisfactory preparation containing less than 1 per cent of moisture and free from ammonia may be obtained from J. T. Baker Chemical Co., Phillipsburg, N. J.

<sup>7</sup> "This temperature indicator consists of powdered chloride-iodide of mercury (HgI<sub>2</sub>) inclosed in a sealed glass bulb not over 1 mm. in diameter. This salt is bright red at ordinary temperatures. At 118° C. it turns yellow and melts to a clear dark red liquid at 155° C. It solidifies again at about 148° C. and resumes its red color gradually only in the course of about twenty-four hours. The melting-point temperature, 153° C., is fortunately a temperature very readily obtained and maintained by means of potassium acetate and as the acetate begins to cake and solidify at 160–161° C., there is no danger in this combination of having either too high or too low a temperature without its being unmistakably apparent.

The HgI<sub>2</sub> may be prepared by heating, in a dry state, intimately mixed mercuric chloride

Close the test-tube by means of a rubber stopper carrying an empty narrow "calcium chloride tube" (1.5 cm. by 25 cm., without bulb) as a condenser. Suspend the test-tube and condenser above a micro-burner (see page 403) by means of a burette clamp or some similar device in such a way that they may be easily raised or lowered. Heat gently, using a bottomless beaker or some similar device as a wind shield if needed. The acetate will soon dissolve (two minutes) and the mixture begin to boil. At this point the indicator begins to melt showing that the desired temperature (153–160° C.) has been reached. Continue the boiling in a gentle, even manner for ten minutes at the end of which time the decomposition of the urea is complete. Remove the apparatus from the flame and dilute the contents with 5 c.c. of water.<sup>1</sup> Add an excess of alkali (2 c.c. of a saturated solution of sodium hydroxide or potassium carbonate) and remove the liberated ammonia by means of a strong air current (see page 404). The ammonia may be caught in a 100 c.c. volumetric flask which contains about 35 c.c. of ammonia-free water and 2 c.c. of *N/10* acid. With a strong air current this process requires only about ten minutes. Determine the ammonia colorimetrically against 1 mg. of nitrogen in the form of ammonium sulphate. For the colorimetric procedure see the total nitrogen determination, page 402.

8. **Method of Folin and Denis.**<sup>2</sup>—Sugar interferes with the decomposition of urea. This was formerly believed to be due to the formation of nitrogenous "melanins,"<sup>3</sup> but is more probably due to the formation of definite, stable ureids.<sup>4</sup> This difficulty may be overcome by proper dilution of the urine thus preventing the formation of the ureids. Because of this great dilution the usual titration procedures are inapplicable, and the following colorimetric procedure is suggested:

Dilute 1 c.c. of the urine with 20 to 100 volumes of ammonia-free water and decompose 1 c.c. of this dilute urine with potassium acetate and acetic acid as described under the method of Folin and Pettibone, No. 2, on page 397.

By means of an air current remove the ammonia to a second test-tube which contains about 2 c.c. of water and 0.5 c.c. of *N/10* hydrochloric acid. Add to the contents of this tube about 2 c.c. of water and 3 c.c. of the diluted (1:5) Nessler-Winkler solution (page 404). Wash this colored

and mercuric iodide in molecular proportions at 150–160° C. for 6–8 hours. At the end of the heating the product should be powdered and used as it is for it cannot be purified by the use of solvents. It should be kept dry until sealed up as indicated." These temperature indicators may be obtained ready prepared in tubes from Eimer & Amend, New York.

<sup>1</sup> This water should be added by means of a pipette through the calcium chloride tube so as to rinse the sides of the tube and the bottom of the rubber stopper from any possible traces of ammonium acetate. Not more than 5 c.c. of water should be used for this purpose.

<sup>2</sup> Folin and Denis; *Jour. Biol. Chem.*, 11, 520, 1912.

<sup>3</sup> Mörner; *Skand. Arch. Physiol.*, 14, 319.

<sup>4</sup> Folin; *Am. Jour. Physiol.*, 13, 46, 1905.

solution into a 10 c.c. volumetric flask and dilute it to the mark with ammonia-free water. Transfer the entire volume to a dry cylinder of a Duboscq colorimeter and determine the depth of color against a standard containing 1 mg. of nitrogen per 100 c.c. of solution. For the detailed colorimetric procedure see the method for total nitrogen, page 402.

## V. Ammonia.

1. **Folin's Method.**—Place 25 c.c. of urine in an aerometer cylinder, 30–40 cm. in height (Fig. 127, below), add about 1 gram of dry sodium carbonate and introduce some crude petroleum to prevent foaming. Insert into the neck of the cylinder a rubber stopper provided with two

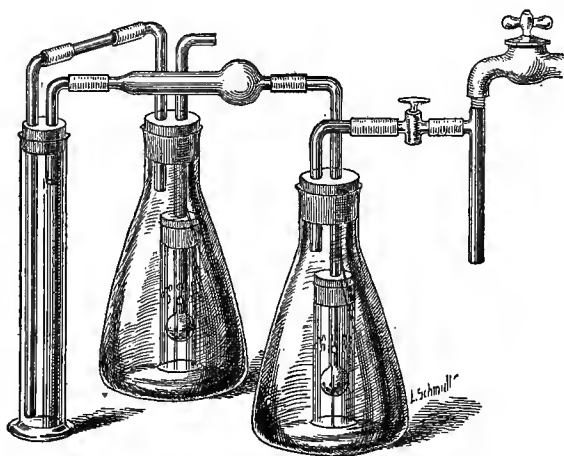


FIG. 127.—FOLIN'S AMMONIA APPARATUS.

perforations, into each of which passes a glass tube, one of which reaches below the surface of the liquid. The shorter tube (10 cm. in length) is connected with a calcium chloride tube filled with cotton, and this tube is in turn joined to a glass tube extending to the bottom of a 500 c.c. wide-mouthed flask which is intended to absorb the ammonia and for this purpose should contain 20 c.c. of  $N/10$  sulphuric acid, 200 c.c. of ammonia-free distilled water and a few drops of an indicator (alizarin red or congo red). To insure the complete absorption of the ammonia the absorption flask is provided with a Folin improved absorption tube (Fig. 128, p. 400) which is very effective in causing the air passing from the cylinder to come into intimate contact with the acid in the absorption flask. In order to exclude any error due to the presence of ammonia in the air a similar absorption apparatus to the one just described is attached to the other side of the aerometer cylinder, thus insuring the passage of *ammonia-free*

air into the cylinder. With an ordinary filter pump and good water pressure the last trace of ammonia should be removed from the cylinder in about one and one-half hours.<sup>1</sup> The number of cubic centimeters of the  $N/10$  sulphuric acid neutralized by the ammonia of the urine may be determined by direct titration with  $N/10$  sodium hydroxide.

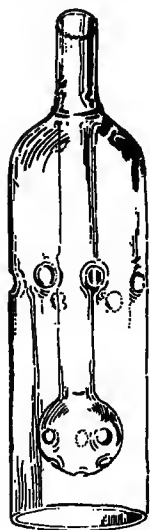


FIG. 128.—FOLIN IMPROVED ABSORPTION TUBE.

This is one of the most satisfactory methods yet devised for the determination of ammonia. Steele<sup>2</sup> has recently suggested a modification for use on urines containing triple phosphate sediments. In this modification .05–1.0 of NaOH and about 15 grams of NaCl are substituted for the  $Na_2CO_3$  of the Folin method.

*Calculation.*—Subtract the number of cubic centimeters of  $N/10$  sodium hydroxide used in the titration from the number of cubic centimeters of  $N/10$  sulphuric acid taken. The remainder is the number of cubic centimeters of  $N/10$  sulphuric acid neutralized by the  $NH_3$  of the urine. 1 c.c. of  $N/10$  sulphuric acid is equivalent to 0.0017 gram of  $NH_3$ . Therefore if  $y$  represents the volume of urine used in the determination and  $y'$  the number of cubic centimeters of  $N/10$  sulphuric acid neutralized by the  $NH_3$  of the urine, we have the following proportion:

$$y : 100 :: y' \times 0.0017 : x \text{ (percentage of } NH_3 \text{ in the urine examined).}$$

Calculate the quantity of  $NH_3$  in the twenty-four-hour urine specimen.

2. **Method of Folin and Macallum.**<sup>3</sup>—By means of Ostwald pipettes (page 403) introduce 1–5 c.c. of urine<sup>4</sup> into a Jena test-tube (20–25 mm. by 200 mm.) and add to the urine a few drops of a solution containing 10 per cent of potassium carbonate and 15 per cent of potassium oxalate. To prevent foaming add a few drops of kerosene or heavy, crude machine oil. Pass a strong air current (see page 404) through the mixture until the ammonia has been entirely removed.<sup>5</sup> Collect the ammonia in a 100 c.c. volumetric flask containing about 20 c.c. of ammonia-free water and 2 c.c. of  $N/10$  acid.

<sup>1</sup> With any given filter pump a "check" test should be made with urine or better with a solution of an ammonium salt of known strength to determine how long the air current must be maintained to remove all the ammonia from 25 c.c. of the solution.

<sup>2</sup> Steele: *Jour. Biol. Chem.*, 8, 365, 1910.

<sup>3</sup> Folin and Macallum: *Jour. Biol. Chem.* 11, 523, 1912.

<sup>4</sup> The volume of urine taken should contain 0.75–1.5 mg. of ammonia nitrogen. With normal urines 2 c.c. will generally yield the desired amount. With very dilute urines 5 c.c. may be required, while with diabetic urines rich in ammonium salts 1 c.c. may be excessive, thus requiring dilution.

<sup>5</sup> Ordinarily a period of ten minutes is sufficiently long.

Nesslerize as described in the method for total nitrogen, page 402, and compare with 1 mg. of nitrogen obtained from a standard ammonium sulphate solution and similarly Nesslerized.

It has been noted that a trace of something capable of giving a color with the Nessler-Winkler solution continues to come long after all the ammonia has been removed from the urine. The nature of this substance has not yet been determined. In actual determinations, by this method, the influence of this unknown substance, because of the small volume of urine used, is entirely negligible.

## VI. Total Nitrogen.

1. **Kjeldahl Method.**<sup>1</sup>—The principle of this method is the conversion of the various nitrogenous bodies of the urine into ammonium sulphate by boiling with concentrated sulphuric acid, the subsequent decomposition of the ammonium sulphate by means of a fixed alkali (NaOH) and the collection of the liberated ammonia in an acid of known strength. Finally, this partly neutralized acid solution is titrated with an alkali of known strength and the nitrogen content of the urine under examination computed.

The procedure is as follows: Place 5 c.c. of urine in a 500 c.c. long-necked Jena glass Kjeldahl flask, add 20 c.c. of concentrated sulphuric acid and about 0.2 gram of copper sulphate and boil the mixture for some time after it is colorless (about one hour). Allow the flask to cool and dilute the contents with about 200 c.c. of ammonia-free water. Add a little more of a concentrated solution of NaOH than is necessary to neutralize the sulphuric acid<sup>2</sup> and introduce into the flask a little coarse pumice stone or a few pieces of granulated zinc,<sup>3</sup> to prevent bumping, and a small piece of paraffin to lessen the tendency to froth. By means of a safety-tube connect the flask with a condenser so arranged that the delivery-tube passes into a vessel containing a known volume (the volume used depending upon the nitrogen content of the urine) of N/10 sulphuric acid, using care that the end of the delivery-tube reaches beneath the surface of the fluid.<sup>4</sup> Mix the contents of the distillation flask very thoroughly by shaking and distil the mixture until its volume has diminished about one-half. Titrate the partly neutralized N/10 sulphuric acid solution by

<sup>1</sup> There are numerous modifications of the original Kjeldahl method; the one described here, however, has given excellent satisfaction and is recommended for the determination of the nitrogen content of urine.

<sup>2</sup> This concentrated sodium hydroxide solution should be prepared in quantity and "check" tests made to determine the volume of the solution necessary to neutralize the volume (20 c.c.) of concentrated sulphuric acid used.

<sup>3</sup> Powdered zinc may be substituted.

<sup>4</sup> This delivery-tube should be of large caliber in order to avoid the "sucking back" of the fluid.

means of N/10 sodium hydroxide, using congo red as indicator, and calculate the content of nitrogen of the urine examined.

*Calculation.*—Subtract the number of cubic centimeters of N/10 sodium hydroxide used in the titration from the number of cubic centimeters of N/10 sulphuric acid taken. The remainder is equivalent to the number of cubic centimeters of N/10 sulphuric acid, *neutralized by the ammonia of the urine*. One c.c. of N/10 sulphuric acid is equivalent to 0.0014 gram of nitrogen. Therefore, if  $y$  represents the volume of urine used in the determination, and  $y'$  the number of cubic centimeters of N/10 sulphuric acid *neutralized by the ammonia of the urine*, we have the following proportion:

$y:100::y' \times 0.0014 : x$  (percentage of nitrogen in the urine examined).

Calculate the quantity of nitrogen in the twenty-four-hour urine specimen.

*Calculation of Percentage Nitrogen Distribution.*—In modern metabolism studies where the various forms of nitrogen are determined, in addition to the total nitrogen as yielded by the Kjeldahl method, it is customary to indicate what portion of the total nitrogen was present in the form of each of the individual nitrogenous constituents. These percentage values are secured by dividing the weight (grams) of nitrogen excreted for the day in the form of each individual nitrogenous constituent by the weight of the total nitrogen output for the same period. For example, if the total nitrogen excretion is 9.814 grams and the excretion of urea-nitrogen is 8.520 grams and the excretions of nitrogen in the forms of ammonia and creatinine are 0.271 gram and 0.639 gram respectively, the percentage distribution for these forms of nitrogen would be calculated as follows:

8.520 grams urea-nitrogen	÷	9.814 grams total nitrogen	=	84.3 per cent
0.271 gram ammonia-nitrogen	÷	9.814 grams total nitrogen	=	2.7 per cent
0.639 gram creatinine-nitrogen	÷	9.814 grams total nitrogen	=	6.5 per cent

2. **Kjeldahl-Folin-Farmer Colorimetric method.**<sup>1</sup>—This method may be considered as a *microchemical* method based on the Kjeldahl-Gunning process for decomposing nitrogenous materials and on the methods of Nessler and of Folin for the determination of ammonia (see page 399). In the regular Kjeldahl procedure 30–100 mg. of nitrogen is manipulated whereas in this modification *only about 1 mg. is utilized*. The method is as follows:

Introduce 5 c.c. of urine into a 50 c.c. volumetric flask if the specific gravity of the urine is over 1018, or into a 25 c.c. flask if the specific gravity is less than 1018.<sup>2</sup> Fill the flask to the mark with distilled water

<sup>1</sup> Folin and Farmer: *Jour. Biol. Chem.*, 11, 493, 1912.

<sup>2</sup> The purpose is to so dilute the urine that 1 c.c. of the diluted fluid shall contain 0.75–1.5 mg. of nitrogen.

and invert it several times in order to guarantee thorough mixing. Transfer *one* cubic centimeter<sup>1</sup> of the diluted urine to a large (20–25 mm. × 200 mm.) Jena-glass test-tube. Add to this 1 c.c. of concentrated sulphuric acid, 1 gram of potassium sulphate, 1 drop of 5 per cent copper sulphate solution and a small, clean, quartz pebble or glass bead. (The pebble or bead is added to prevent bumping.) Boil the mixture over a micro-burner<sup>2</sup> for about *six minutes*, *i. e.*, about two minutes after the

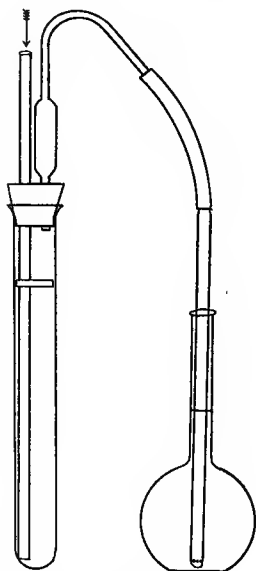


FIG. 129.

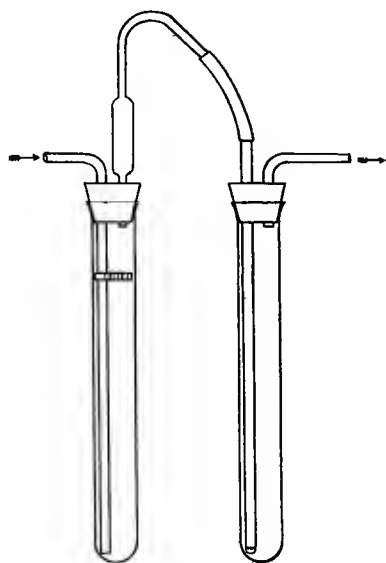


FIG. 130.

FIGS. 129 and 130.—Forms of Apparatus used in Methods of Folin and Associates for Determination of Total Nitrogen, Urea and Ammonia. (From *Jour. Biol. Chem.*, vol. 11, 1912.)

mixture has become colorless. Allow to cool until the digestion mixture begins to become viscous. This ordinarily takes about three minutes, but in any event the mixture *must not be permitted to solidify*. Add about 6 c.c. of water (a few drops at a time, at first, then more rapidly) to prevent solidification. To this acid solution add an excess of sodium hydroxide (3 c.c. of a *saturated* solution is sufficient) and aspirate the liberated ammonia by means of a rapid air current<sup>3</sup> into a volumetric

<sup>1</sup> This measurement should be made by means of a modified Ostwald pipette (see Ostwald-Luther: *Physiko-Chemische Messungen*, 2d. ed., p. 135). Such pipettes may be obtained from Eimer and Amend, New York.

<sup>2</sup> A type of burner which has proven satisfactory is Eimer and Amend's No. 2587.

<sup>3</sup> Either a vacuum pump or compressed air or a force pump may be used. The compressed air method is rather the more convenient inasmuch as the ammonia may be collected directly in a volumetric flask. Inasmuch as the necks of such flasks (100 c.c.) are not large enough to permit of the use of a two-hole rubber stopper when suction is used, the ammonia should be collected in one of the Jena test-tubes previously described which contains 2 c.c. of N/10 hydrochloric acid and about 5 c.c. of ammonia-free water. The ammonium salt is then transferred to the volumetric flask with 40–50 c.c. of water and Nesslerized as described.

flask (100 c.c.) containing about 20 c.c. of ammonia-free water and 2 c.c. of N/10 hydrochloric acid. (See Figs. 129 and 130, p. 403.) The air current should be only moderately rapid for the first two minutes but at the end of this two-minute period the current should be run at its maximum speed for an interval of *eight minutes*.

Disconnect the flask, dilute the contents to about 60 c.c. with ammonia-free water and dilute similarly 1 mg. of nitrogen in the form of ammonium sulphate<sup>1</sup> in a second volumetric flask. Nesslerize both solutions as nearly as possible at the same time with 5 c.c. of Nessler-Winkler solution<sup>2</sup> diluted, *immediately before using*, with about 25 c.c. of ammonia-free water to avoid turbidity. Immediately fill the two flasks to the mark with ammonia-free water, mix well and determine the relative intensity of the two colors by means of a Duboscq colorimeter.<sup>3</sup>

The color of the unknown should be adjusted to that of the standard both from *above* and *below* the level of the latter. The matching of the colors is ordinarily very easy. It is desirable to make the readings by diffused daylight if possible. If electric light must be used, a sheet of smooth white paper should be interposed between the colorimeter and the source of light.

*Calculation.*—The reading of the standard divided by the reading of the unknown gives the *nitrogen in milligrams in the volume of the urine taken*. Calculate the total nitrogen output for the twenty-four-period.

## VII. Amino Nitrogen.

**Van Slyke's Method.**<sup>4</sup>—The method is based on the fact that nitrous acid in solution spontaneously decomposes with formation of nitric oxide:



<sup>1</sup>Care should be taken to secure the pure salt. All ammonium salts contain pyridine bases which titrate like ammonia but do not react with Nessler's reagent. *Pure* ammonium sulphate may be prepared by decomposing a high-grade ammonium salt with sodium hydroxide and passing the liberated ammonia into pure sulphuric acid. The salt is then precipitated by means of alcohol, then brought into solution in water and re-precipitated by alcohol. The final product should be dried in a desiccator over sulphuric acid. Dr. H. L. Emerson of Boston prepares a salt which is very satisfactory for use in this method.

<sup>2</sup>*Chem. Zeit.*, 1899, p. 541. The Nessler-Winkler solution has the following formula:

Mercuric iodide.....	10 grams.
Potassium iodide.....	5 grams.
Sodium hydroxide.....	20 grams.
Water.....	100 c.c.

The mercuric iodide is rubbed up in a small porcelain mortar with water, then washed into a flask and the potassium iodide added. The sodium hydroxide is dissolved in the remaining water and the cooled solution added to the above mixture. The solution cleared by standing is preserved in a dark bottle.

The 25 c.c. portion of the diluted reagent should be added about one-third at a time to the contents of the flask. It is very essential that the dilution of the reagent takes place *immediately preceding its use*, inasmuch as the diluted reagent deteriorates in a few minutes as is indicated by the formation of a brick-red precipitate. Fortunately the reagent does not decompose in this manner in the presence of the ammonium salt.

<sup>3</sup>The standard may be set at any desired depth but a very satisfactory depth is 20 mm. The depth should be uniform throughout any series of comparative tests.

<sup>4</sup>Van Slyke: *Jour. Biol. Chem.*, 9, 185, 1911.



This reaction is utilized in displacing all the air of the apparatus with nitric oxide. The amino solution is then introduced, evolution of nitrogen mixed with nitric oxide resulting. The oxide is absorbed by alkaline permanganate solution, and the pure nitrogen measured in a special gas burette shown in the figure.

The determination of amino nitrogen is carried out as follows: 50 c.c. of the total day's urine are measured into a flask and 2 c.c. of concentrated acid added. The acid urine is then placed in an autoclave and

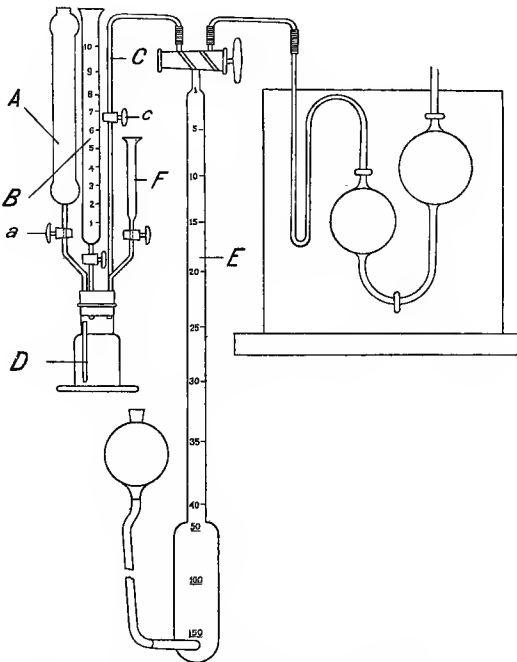


FIG. 131.—VAN SLYKE'S AMINO NITROGEN APPARATUS.

heated to  $175^{\circ}$  C. under pressure for an hour and a half. After hydrolysis is complete, a few drops of sodium alizarin sulphonate are added as indicator and potassium hydroxide added in such quantity as to leave an excess of the reagent. The solution is then boiled 20–30 minutes in order to get rid of all the ammonia. After boiling, the solution is approximately neutralized and the volume made up exactly to 50 c.c. Ten cubic centimeters of this is then used for each amino acid determination.

The 10 c.c. of urine treated as described are contained in the burette (B) of the van Slyke apparatus (see Fig. 131). The detailed manipulation of the urine in order to determine the amino acid content is very simple to follow. Into the reaction chamber (D) one pours 28 c.c. of sodium ni-

trite solution (30 grams to 100 c.c. of water) and 7 c.c. of glacial acetic acid. The stopper is then inserted in (D), and the *three-way* stop-cock (c) opened so as to allow the gases to escape into the air. 5 c.c. of water are now placed in vessel (A) and allowed to run into (D) so as to expell the air remaining in the apparatus. The cock (c) is closed, (a) left open and the solution from (D) allowed to back up into (A) until about 5 c.c. are accumulated there, (D) being shaken so as to get rid of any air dissolved in the interacting solutions. The gases are again expelled as described by opening (c). This process is repeated thus washing the last traces of air from the apparatus. (a) is now opened, (c) closed and about 25 c.c. of the solution forced into (A) by the pressure of the nitric oxide formed in (D). The cock (c) is then opened so that the gas passes into the burette (F), (a) closed, and the 10 c.c. of urine run into (D). After the reaction has run for five minutes, (D) is shaken and the remainder of the gas forced from (D) into the burette by allowing the liquid in (A) to run into (D). The gases are then run from the burette into the pipette (H), the latter is thoroughly shaken, till no more gas is absorbed by the alkaline permanganate solution.<sup>1</sup> The pure nitrogen gas is run back into the burette and measured. The temperature of the gas and barometric pressure are recorded. Blanks should be run and the slight error due to the formation of nitrogen gas and oxygen from the interaction of sodium nitrite and glacial acetic acid accounted for.<sup>2</sup>

*Calculation.*—As the reaction doubles the amount of nitrogen present as amino nitrogen, the volume of nitrogen found must be divided by 2. The results are expressed in milligrams of nitrogen.

### VIII. Hippuric Acid.

**Dakin's Methods.**<sup>3</sup> *Preliminary Procedure.*—Place 150 c.c. (or more) of the urine under examination in a porcelain evaporating dish and evaporate *almost* to dryness upon a water-bath. Add about 1 gram of sodium dihydrogen phosphate, about 25 grams of gypsum ( $\text{CaSO}_4, 2\text{H}_2\text{O}$ ) and rub up with a pestle and stir with a spatula until a uniform mixture results. Dry the powder thus produced in a water-oven for about two hours, at the end of which period it should be rubbed up a second time, to remove lumps, and transferred to a Schleicher and Schüll "extraction shell" and extracted in a Soxhlet apparatus in the usual way

<sup>1</sup> The alkaline permanganate solution contains 50 grams of potassium permanganate and 25 grams of potassium hydroxide per liter.

<sup>2</sup> According to Robinson (Mich. Ag. Exp. Station Tech. Bull., 7, p. 11, 1911), analysis of the gases formed by the decomposition of sodium nitrite with glacial acetic acid indicates the presence of small amounts of free oxygen and nitrogen.

<sup>3</sup> Private communication to the author from Dr. H. D. Dakin.

(see p. 437). The extraction medium is ethyl acetate and the flask containing the acetate should be strongly heated over a *sand-bath*<sup>1</sup> for about two hours. The ethyl acetate extract is now transferred to a separatory funnel, and the original flask rinsed with sufficient fresh ethyl acetate to make the total volume in the separatory funnel<sup>2</sup> about 100 c.c. Wash the ethyl acetate solution *five times* with a saturated solution of sodium chloride, using 8 c.c. of the sodium chloride solution at each extraction, shaking vigorously and removing the sodium chloride extract in each case before adding fresh sodium chloride solution. The sodium chloride *removes the urea completely* and the hippuric acid is then determined in the urea-free solution by the following volumetric or gravimetric procedure:

1. *Volumetric Determination.*—Transfer the urea-free ethyl acetate solution, prepared as described above, to a Kjeldahl flask, add about 25 c.c. of water, a small piece of pumice stone to prevent bumping, attach a condenser and distil off the acetate<sup>3</sup> over a free flame. After practically all of the ethyl acetate has been distilled off, the nitrogen in the remaining solution should be determined by means of the Kjeldahl method (see p. 402).

The main source of error in this method is the fact that any nitrogen present in the form of *phenaceturic acid* or *indole acetic acid* is determined as hippuric acid nitrogen. The error from this source is, however, usually trifling.

*Calculation.*—Calculate as usual for nitrogen determinations, remembering that 1 c.c. of N/10 sulphuric acid is equivalent to 0.0179 gram hippuric acid.

2. *Gravimetric Determination.*—The urea-free ethyl acetate solution, contained in the separatory funnel, after washing with sodium chloride solution, as described under Preliminary Procedure, p. 406, is washed with 5 c.c. of distilled water to remove the major portion of the sodium chloride. Transfer the solution from the separatory funnel to a round-bottomed flask and subject it to a steam distillation in the usual way. A *slow* current of steam should be used while the ethyl acetate is being distilled off and later a more rapid current may be employed. The distillation should be continued for twenty minutes. Now add about 0.1 gram of charcoal to the aqueous solution which is heated to boiling and filtered *hot*. Evaporate the solution in a *weighed* Jena glass dish on a water-bath until the volume of the solution is reduced to about 3 c.c. Stand the dish in a warm place until evaporation is complete

<sup>1</sup> A water-bath cannot be substituted inasmuch as the resultant extraction would be *too slow*.

<sup>2</sup> This ethyl acetate solution contains hippuric acid, urea, and other substances.

<sup>3</sup> The ethyl acetate after separation from the watery layer of the distillate may be dried over calcium chloride and used again.

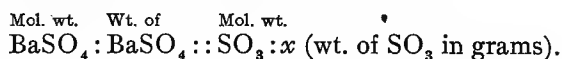
and a crystalline residue remains. Wash the residue, in turn, with 2 c.c. of dry ether, and 1 c.c. of water, dry it in an air-bath at 100° C. and weigh. If it is so desired the residue may be recrystallized from a little hot water and the melting-point determined. Pure hippuric acid melts at 187° C. Contamination with phenaceturic acid may be detected both by the melting-point and the microscopical characteristics.

## IX. Sulphur.

1. **Total Sulphates.** *Folin's Method.*—Place 25 c.c. of urine in a 200–250 c.c. Erlenmeyer flask, add 20 c.c. of dilute hydrochloric acid<sup>1</sup> (1 volume of concentrated HCl to 4 volumes of water) and gently boil the mixture for 20–30 minutes. To minimize the loss of water by evaporation the mouth of the flask should be covered with a small watch glass during the boiling process. Cool the flask for 2–3 minutes in running water, and dilute the contents to about 150 c.c. by means of cold water. Add 10 c.c. of a 5 per cent solution of barium chloride slowly, drop by drop, to the cold solution.<sup>2</sup> The contents of the flask *should not be stirred or shaken* during the addition of the barium chloride. Allow the mixture to stand at least one hour, then shake up the solution and filter it through a weighed Gooch crucible.<sup>3</sup>

Wash the precipitate of BaSO<sub>4</sub> with about 250 c.c. of cold water, dry it in an air-bath or over a very low flame, then ignite,<sup>4</sup> cool and weigh.

*Calculation.*—Subtract the weight of the Gooch crucible from the weight of the crucible and the BaSO<sub>4</sub> precipitate to obtain the weight of the precipitate. The weight of SO<sub>3</sub><sup>5</sup> in the volume of urine taken may be determined by means of the following proportion.



<sup>1</sup> If it is desired, 50 c.c. of urine and 4 c.c. of concentrated acid may be used instead.

<sup>2</sup> A dropper or capillary funnel made from an ordinary calcium chloride tube and so constructed as to deliver 10 c.c. in 2–3 minutes is recommended for use in adding the barium chloride.

<sup>3</sup> If a Gooch crucible is not available, the precipitate of BaSO<sub>4</sub> may be filtered off upon a washed filter paper (Schleicher & Schüll's, No. 589, blue ribbon), and after washing the precipitate with about 250 c.c. of cold water the paper and precipitate may be dried in an air-bath or over a low flame. The ignition may then be carried out in the usual way in the ordinary platinum or porcelain crucible. In this case correction must be made for the weight of the ash of the filter paper used.

<sup>4</sup> Care must be taken in the ignition of precipitates in Gooch crucibles. The flame should never be applied directly to the perforated bottom or to the sides of the crucible, since such manipulation is invariably attended by mechanical losses. The crucibles should always be provided with lids and tight bottoms during the ignition. In case porcelain Gooch crucibles, whose bottoms are not provided with a non-perforated cap, are used, the crucible may be placed upon the lid of an ordinary platinum crucible during ignition. The lid should be supported on a triangle, the crucible placed upon the lid and the flame applied to the improvised bottom. Ignition should be complete in 10 minutes if no organic matter is present.

<sup>5</sup> It is considered preferable by many investigators to express all sulphur values in terms of S rather than SO<sub>3</sub>.

Representing the weight of the  $\text{BaSO}_4$  precipitate by  $y$  and substituting the proper molecular weights, we have the following proportion:

$231.7:y::79.5:x$  (wt. of  $\text{SO}_3$  in grams in the quantity of urine used).

Calculate the quantity of  $\text{SO}_3$  in the twenty-four-hour specimen of urine.

To express the result in *percentage* of  $\text{SO}_3$  simply divide the value of  $x$ , as just determined, by the quantity of urine used.

**2. Inorganic Sulphates.** *Folin's Method.*—Place 25 c.c. of urine and 100 c.c. of water in a 200–250 c.c. Erlenmeyer flask and acidify the diluted urine with 10 c.c. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). In case the urine is dilute 50 c.c. may be used instead of 25 c.c. and the volume of water reduced proportionately. Add 10 c.c. of 5 per cent barium chloride slowly, drop by drop, to the cold solution and from this point proceed as indicated in the method for the determination of Total Sulphates, page 408.

Calculate the quantity of inorganic sulphates, expressed as  $\text{SO}_3$ , in the twenty-four-hour urine specimen.

*Calculation.*—Calculate according to the directions given under Total Sulphates, above.

**3. Ethereal Sulphates.** *Folin's Method.*—Place 125 c.c. of urine in an Erlenmeyer flask of suitable size, dilute it with 75 c.c. of water and acidify the mixture with 30 c.c. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). To the *cold* solution add 20 c.c. of a 5 per cent solution of barium chloride, drop by drop.<sup>1</sup> Allow the mixture to stand about one hour, then filter it through a dry filter paper.<sup>2</sup> Collect 125 c.c. of the filtrate and boil it gently for at least one-half hour. Cool the solution, filter off the precipitate of  $\text{BaSO}_4$ , wash, dry and ignite it according to the directions given on page 408.

*Calculation.*—The weight of the  $\text{BaSO}_4$  precipitate should be multiplied by 2 since only one-half (125 c.c.) of the total volume (250 c.c.) of fluid was precipitated by the barium chloride. The remaining calculation should be made according to directions given under Total Sulphates, page 408.

Calculate the quantity of ethereal sulphates, expressed as  $\text{SO}_3$ , in the twenty-four-hour urine specimen.

**4. Total Sulphur.** *Benedict's Method.*<sup>3</sup>—Ten cubic centimeters of urine are measured into a *small* (7–8 cm.) porcelain evaporating dish and

<sup>1</sup> See note (2) at the bottom of page 408.

<sup>2</sup> This precipitate consists of the inorganic sulphates. If it is desired, this  $\text{BaSO}_4$  precipitate may be collected in a Gooch crucible or on an ordinary quantitative filter paper and a determination of inorganic sulphates made, using the same technic as that suggested above. In this way we are enabled to determine the inorganic and ethereal sulphates in the same sample of urine.

<sup>3</sup> Benedict: *Journal of Biological Chemistry*, 6, 363, 1909.

5 c.c.<sup>1</sup> of Benedict's sulphur reagent<sup>2</sup> added. The contents of the dish are evaporated over a free flame which is regulated to keep the solution just below the boiling-point, so that there can be no loss through spattering. When dryness is reached the flame is raised slightly until the entire residue has blackened. The flame is then turned up in two stages to the full heat of the bunsen burner and the contents of the dish thus *heated to redness for ten minutes after the black residue (which first fuses) has become dry*. This heating is to decompose the last traces of nitrate (and chlorate). The flame is then removed and the dish allowed to cool more or less completely. Ten to twenty cubic centimeters of dilute (1 : 4) hydrochloric acid is then added to the residue in the dish, which is then warmed gently until the contents have completely dissolved and a perfectly clear, sparkling solution is obtained. This dissolving of the residue requires scarcely two minutes. With the aid of a stirring rod the solution is washed into<sup>3</sup> a small Erlenmeyer flask, diluted with cold, distilled water to 100–150 c.c., 10 c.c. of 10 per cent barium chloride solution added drop by drop, and the solution allowed to stand for about an hour. It is then shaken up and filtered as usual through a weighed Gooch crucible.

*Calculation.*—Make the calculation according to directions given under Total Sulphates, p. 408. Calculate the quantity of sulphur, expressed as  $\text{SO}_3$  or S, present in the twenty-four-hour urine specimen.

5. **Total Sulphur.** *Osborne-Folin Method.*—Place 25 c.c. of urine<sup>4</sup> in a 200–250 c.c. *nickel* crucible and add about 3 grams of sodium peroxide. Evaporate the mixture to a syrup upon a steam water-bath and heat it carefully over an alcohol flame until it solidifies (15 minutes). Now remove the crucible from the flame and allow it to cool. Moisten the residue with 1–2 c.c. of water,<sup>5</sup> sprinkle about 7–8 grams of sodium peroxide over the contents of the crucible and fuse the mass over an alcohol flame for about 10 minutes. Allow the crucible to cool for a few minutes, add about 100 c.c. of water to the contents and heat at least one-half hour over an alcohol flame to dissolve the alkali and decompose the sodium peroxide. Next rinse the mixture into a 400–450 c.c. Erlenmeyer flask, by means of hot water, and dilute it to about 250 c.c. Heat the solution nearly to the boiling-point and add concentrated hydrochloric acid slowly until the nickelic oxide, derived from the crucible, is just

<sup>1</sup> If the urine is concentrated the quantity should be slightly increased.

<sup>2</sup> Crystallized copper nitrate, sulphur-free or of known sulphur content.... 200 grams.  
Sodium or potassium chlorate..... 50 grams.  
Distilled water to..... 1000 C.C.

<sup>3</sup> Sometimes the porcelain glaze cracks during heating, in which case the solution should be filtered into the flask.

<sup>4</sup> If the urine is very dilute 50 c.c. may be used.

<sup>5</sup> This moistening of the residue with a small amount of water is very essential and should not be neglected.

brought into solution.<sup>1</sup> A few minutes boiling should now yield a *clear* solution. In case too little peroxide or too much water was added for the final fusion a clear solution will not be obtained. In this event cool the solution and remove the insoluble matter by filtration.

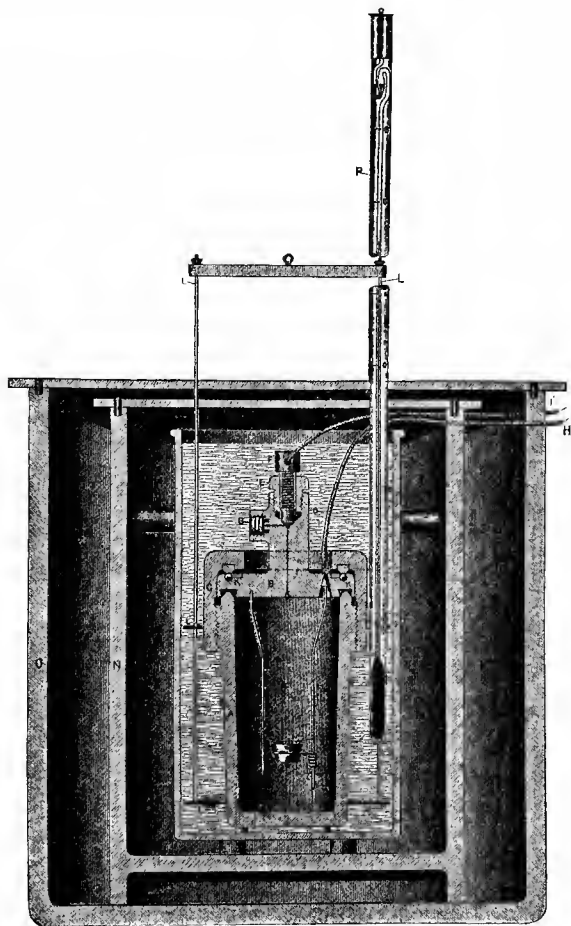


FIG. 132.—BERTHELOT-ATWATER BOMB CALORIMETER. (CROSS-SECTION OF APPARATUS AS READY FOR USE.)

A, Steel cup or bomb proper; C, collar of steel; G, opening through which oxygen is forced into the bomb; H and H', insulated wires which serve to conduct an electric current for igniting the substance which is held in the small capsule; L, a stirrer which serves to keep the water surrounding the bomb in motion and insures the equalization of temperature; P, a delicate thermometer which shows the rise in temperature of the water surrounding the bomb.

To the clear solution add 5 c.c. of very dilute alcohol (about 18–20 per cent) and continue the boiling for a few minutes. The alcohol is added to remove the chlorine which was formed when the solution was

<sup>1</sup> About 18 c.c. of acid is required for 8 grams of sodium peroxide.

acidified. Add 10 c.c. of a 10 per cent solution of barium chloride, slowly, drop by drop,<sup>1</sup> to the liquid. Allow the precipitated solution to stand in the cold *two days* and then filter and continue the manipulation according to the directions given under Total Sulphates, page 408.

*Calculation.*—Make the calculation according to directions given under Total Sulphates, p. 408. Calculate the quantity of sulphur, expressed as  $\text{SO}_3$  or S, present in the twenty-four-hour urine specimen.

**6. Total Sulphur.**—*Sodium Hydroxide and Potassium Nitrate Fusion Method.*—Place 25 c.c. of urine in a *silver* crucible and evaporate to a thick syrup on a water-bath. Add 10 grams of sodium hydroxide and 2 grams of potassium nitrate to the residue and fuse the mass, over an alcohol flame, until all organic matter has disappeared and the fused mixture is clear. Cool the mixture, transfer it to a casserole by means of hot water, acidify slightly with hydrochloric acid and evaporate it to dryness on a water-bath. Moisten the residue with a few drops of dilute hydrochloric acid and bring it into solution with hot water. Filter, heat the filtrate to boiling, and immediately precipitate it by the addition of 10 c.c. of a 10 per cent solution of barium chloride, adding the solution slowly, drop by drop. Allow the precipitated solution to stand 2 hours and filter while *cold*. Ignite, weigh, and calculate according to directions given under Total Sulphates, p. 408.

Compute the quantity of sulphur, expressed as  $\text{SO}_3$  or S, present in the twenty-four-hour urine specimen.

**7. Total Sulphur.** *Sherman's Compressed Oxygen Method.*<sup>2</sup>—Evaporate as much urine on an absorbent filter block<sup>3</sup> at 55° C. as the block will conveniently absorb and burn the block so prepared in a bomb-calorimeter<sup>4</sup> using 25–30 atmospheres of oxygen. Connect the bomb with a wash-bottle containing water, and allow the gas to bubble through the liquid until the high pressure within the apparatus has been reduced to atmospheric pressure. Now open the bomb and thoroughly rinse the interior, using water from the wash-bottle for the first rinsing. Dissolve any ash found in the combustion capsule in hydrochloric acid and add this solution to the main solution. Evaporate to 150 c.c., filter, and cool the filtrate. Add 10 c.c. of a 5 per cent solution of barium chloride to the *cold* filtrate, slowly, drop by drop.<sup>5</sup> The contents of the flask should not be stirred or shaken during the addition of the barium chloride. Allow the mixture to stand at least one hour, then shake up the solution and

<sup>1</sup> See note (2) at the bottom of page 408.

<sup>2</sup> See Sherman's Organic Analysis, First edition, p. 19.

<sup>3</sup> Only a small amount of urine should be added at one time, it being necessary to make several evaporations before the block contains sufficient urinary residue to proceed with the combustion.

<sup>4</sup> The Berthelot-Atwater apparatus (Fig. 132, page 411) is well adapted to this purpose.

<sup>5</sup> See note (2) at the bottom of page 408.



filter it through a weighed Gooch crucible. Manipulate the precipitate of  $\text{BaSO}_4$  according to directions given under Total Sulphates, page 408.

Calculate the quantity of sulphur, expressed as  $\text{SO}_3$  or S, present in the twenty-four-hour urine specimen.

## X. Phosphorus.

1. **Total Phosphates.** *Uranium Acetate Method.*—To 50 c.c. of urine in a small beaker or Erlenmeyer flask add 5 c.c. of a special sodium acetate solution<sup>1</sup> and heat the mixture to the boiling-point. From a burette, run into the hot mixture, drop by drop, a standard solution of uranium acetate<sup>2</sup> until a precipitate ceases to form and a drop of the mixture when removed by means of a glass rod and brought in contact with a drop of a solution of potassium ferrocyanide on a porcelain test-tablet produces instantaneously a brownish-red coloration.<sup>3</sup> Take the burette reading and calculate the  $\text{P}_2\text{O}_5$  content of the urine under examination.

*Calculation.*—Multiply the number of cubic centimeters of uranium acetate solution used by 0.005 to determine the number of *grams* of  $\text{P}_2\text{O}_5$  in the 50 c.c. of urine used. To express the result in *percentage* of  $\text{P}_2\text{O}_5$  multiply the value just obtained by 2, *e. g.*, if 50 c.c. of urine contained 0.074 gram of  $\text{P}_2\text{O}_5$  it would be equivalent to 0.148 per cent.

Calculate, in terms of  $\text{P}_2\text{O}_5$ , the total phosphate content of the twenty-four-hour urine specimen.

2. **Earthy Phosphates.**—To 100 c.c. of urine in a beaker add an excess of ammonium hydroxide and allow the mixture to stand 12–24 hours. Under these conditions the phosphoric acid in combination with the alkaline earths, calcium and magnesium, is precipitated as phosphates of these metals. Collect the precipitate on a filter paper and wash it with very dilute ammonium hydroxide. Pierce the paper, and remove the precipitate by means of hot water. Bring the phosphates into solution by adding a small amount of dilute acetic acid to the warm solution. Make the volume up to 50 c.c. with water, add 5 c.c. of sodium acetate

<sup>1</sup> The sodium acetate solution is prepared by dissolving 100 grams of sodium acetate in 800 c.c. of distilled water, adding 100 c.c. of 30 per cent acetic acid to the solution, and making the volume of the mixture up to 1 liter with water.

<sup>2</sup> This uranium acetate solution may be prepared by dissolving about 34 grams of uranium acetate in one liter of water. One c.c. of this solution should now be made equivalent to 0.005 gram of  $\text{P}_2\text{O}_5$ , phosphoric anhydride. It may be standardized as follows: To 50 c.c. of a standard solution of disodium hydrogen phosphate, of such a strength that the 50 c.c. contains 0.1 gram of  $\text{P}_2\text{O}_5$ , add 5 c.c. of the sodium acetate solution mentioned above, and titrate with the uranium solution to the correct end-reaction as indicated in the method proper. Inasmuch as 1 c.c. of the uranium solution should precipitate 0.005 gram of  $\text{P}_2\text{O}_5$ , exactly 20 c.c. of the uranium solution should be required to precipitate 50 c.c. of the standard phosphate solution. If the two solutions do not bear this relation to each other they may be brought into proper relation by diluting the uranium solution with distilled water or by increasing its strength.

<sup>3</sup> A 10 per cent solution of potassium ferrocyanide is satisfactory.

solution, and determine the  $P_2O_5$  content of the mixture according to the directions given under the previous method.

*Calculation.*—Multiply the number of cubic centimeters of uranium acetate solution used by 0.005 to determine the number of *grams* of  $P_2O_5$  in the 100 c.c. of urine used. Since 100 c.c. of urine was taken this value also expresses the *percentage* of  $P_2O_5$  present.

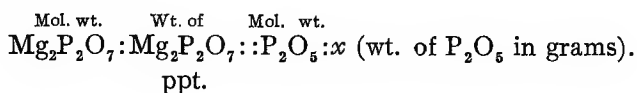
Calculate the quantity of earthy phosphates, in terms of  $P_2O_5$ , present in the twenty-four-hour urine specimen.

The quantity of phosphoric acid present in combination with the *alkali* metals may be determined by subtracting the content of earthy phosphates from the total phosphates.

**Total Phosphorus.**—*Sodium Hydroxide and Potassium Nitrate Fusion Method.*—Place 25 c.c. of urine in a large silver crucible and evaporate to a syrup on a water-bath. Add 10 grams of NaOH and 2 grams of  $KNO_3$  to the residue and fuse the mass until all organic matter has disappeared and the fused mixture is clear. Cool the mixture, transfer it to a casserole by means of hot water, acidify the solution slightly with pure nitric acid, and evaporate to dryness on a water-bath. Moisten the residue with a few drops of dilute nitric acid, dissolve it in hot water, and transfer to a beaker. Now add an equal volume of molybdc solution<sup>1</sup> and keep the mixture at 40° C. for twenty-four hours. Filter off the precipitate, wash it with dilute molybdc solution, and dissolve it in dilute ammonia. Add dilute hydrochloric acid to the solution, being careful to leave the solution distinctly ammoniacal. Magnesia mixture<sup>2</sup> (10–15 c.c.) should now be added and after stirring thoroughly and making strongly ammoniacal with concentrated ammonia the solution should be allowed to stand in a cool place for twenty-four hours. Filter off the precipitate, wash it free from chlorine by means of dilute ammonia (1 : 5), dry, incinerate, and weigh, as magnesium pyrophosphate,  $Mg_2P_2O_7$ , in the usual manner.

In this method the phosphoric acid of the urine is precipitated as *ammonium magnesium phosphate* and in the process of incineration this body is transformed into *magnesium pyrophosphate*.

*Calculation.*—The quantity of phosphorus, expressed in terms of  $P_2O_5$ , in the volume of urine taken may be determined by means of the following proportion:



<sup>1</sup> Directions for the preparation of the solution are given on p. 64.

<sup>2</sup> Directions for the preparation of magnesia mixture may be found on p. 313.

If  $y$  represents the weight of the  $Mg_2P_2O_7$  precipitate and we make the proper substitution we have the following proportion:

221.1 :  $y$  :: 140.9 :  $x$  (wt. of  $P_2O_5$ , in grams, in the quantity of urine used.)

To express the result in *percentage* of  $P_2O_5$ , simply divide the value of  $x$ , as just determined, by the quantity of urine used.

## XI. Creatinine.

**Folin's Colorimetric Method.**—This method is based upon the characteristic property possessed alone by creatinine, of yielding a certain definite color-reaction in the presence of picric acid in alkaline solution. The procedure is as follows: Place 10 c.c. of urine in a 500 c.c. volumetric flask, add 15 c.c. of a *saturated* solution of picric acid and 5 c.c. of a 10 per cent solution of sodium hydroxide, shake thoroughly and allow the mixture to stand for 5 minutes. During this interval pour a little N/2 potassium bichromate solution<sup>1</sup> into each of the two cylinders of the colorimeter (Duboscq's) and carefully adjust the depth of the solution in one of the cylinders to the 8 mm. mark. A few preliminary colorimetric readings may now be made with the solution in the other cylinder, in order to insure greater accuracy in the subsequent examination of the solution of unknown strength. Obviously the two solutions of potassium bichromate are identical in color and in their examination no two readings should differ more than 0.1–0.2 mm. from the true value (8 mm.). Four or more readings should be made in each case and an average taken of all of them *exclusive* of the first reading, which is apt to be less accurate than the succeeding readings. In time as one becomes proficient in the technic it is perfectly safe to take the average of the *first two readings*.

At the end of the 5-minute interval already mentioned, the contents of the 500 c.c. flask are diluted to the 500 c.c. mark, the bichromate solution is thoroughly rinsed out of one of the cylinders, and replaced with the solution thus prepared and a number of colorimetric readings are *immediately* made.

Ordinarily 10 c.c. of urine is used in the determination by this method, but if the content of creatinine is above 15 mg. or below 5 mg. the determination should be repeated with a volume of urine selected according to the content of creatinine. This variation in the volume of urine according to the content of creatinine is quite essential, since the method loses in accuracy when more than 15 mg. or less than 5 mg. of creatinine is present in the solution of unknown strength.

*Calculation.*—By experiment it has been determined that 10 mg.

<sup>1</sup> This solution contains 24.55 grams of potassium bichromate to the liter.

of pure creatinine, when brought into solution and diluted to 500 c.c. as explained in the above method, yields a mixture 8.1 mm. of which possesses the same colorimetric value as 8 mm. of a N/2 solution of potassium bichromate. Bearing this in mind the computation is readily made by means of the following proportion in which  $y$  represents the number of millimeters of the solution of unknown strength equivalent to the 8 mm. of the potassium bichromate solution:

$$y : 8.1 :: 10 : x \text{ (mgs. of creatinine in the quantity of urine used).}$$

This proportion may be used for the calculation no matter what volume of urine (5, 10, or 15 c.c.) is used in the determination. The 10 represents 10 *mg. of creatinine* which gives a color equal to 8.1 mm., whether dissolved in 5, 10, or 15 c.c. of fluid.

Calculate the quantity of creatinine in the twenty-four-hour urine specimen.

## XII. Creatine.

**Folin-Benedict and Myers Method.**<sup>1</sup>—To 20 c.c. of urine in a 50 c.c. volumetric flask, add 20 c.c. of normal hydrochloric acid and place the flask in an autoclave at a temperature of 117–120° C. for one-half hour. Add distilled water until the volume of the acid-urine mixture is exactly 50 c.c., close the flask by means of a stopper, and shake it thoroughly. Approximately neutralize 25 c.c. of this mixture, introduce it into a 500 c.c. volumetric flask and determine its creatinine content according to Folin's Method (see p. 415).

*Calculation.*—Calculate as explained on p. 415, and from this value subtract the value for the original content of creatinine *before hydrolysis*. The difference between these two values will be the creatine content of the original urine *in terms of creatinine*.

## XIII. Indican.

**Ellinger's Method.**—This method for the quantitative determination of indican is based upon the principle underlying Jaffe's test for the *detection* of indican (see p. 298). The method is as follows: To 50 c.c. of urine<sup>2</sup> in a small beaker or casserole add 5 c.c. of basic lead acetate solution,<sup>3</sup> mix thoroughly, and filter. Transfer 40 c.c. of the filtrate to a separatory funnel, add an equal volume of Obermayer's reagent (see p. 299) and 20 c.c. of chloroform, and extract in the usual

<sup>1</sup> Benedict and Myers: *Am. J. Phys.*, 18, 397, 1907.

<sup>2</sup> If the urine under examination is neutral or alkaline in reaction it should be made faintly acid with acetic acid before adding the basic lead acetate.

<sup>3</sup> For preparation of basic lead acetate solution see Appendix.

manner. This extraction with chloroform should be repeated until the chloroform solution remains colorless. Shake up the combined chloroform extracts 2-3 times with distilled water in a separating funnel and complete the purification by extracting with very dilute sodium hydroxide (1:1000). Remove all traces of alkali by washing with water. Now filter the combined chloroform extracts through a *dry* filter paper into a *dry* Erlenmeyer flask. Distil off the chloroform, heat the residue on a boiling water-bath for 5 minutes in the open flask, and wash the dried residue with hot water<sup>1</sup>. Add 10 c.c. of concentrated sulphuric acid to the washed residue, heat on the water-bath for 5-10 minutes, dilute with 100 c.c. of water, and titrate the blue solution with a very dilute solution of potassium permanganate.<sup>2</sup> The end-point is indicated by the dissipation of all the blue color from the solution and the formation of a pale yellow color.

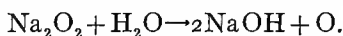
Beautiful plates of indigo blue sometimes appear in the chloroform extract of urines containing abundant indican. In urines preserved by thymol the determination of indican is interfered with unless great care is taken in washing the chloroform extract with dilute alkali. Care should be taken, therefore, to make the indican determination upon fresh urine, before the addition of the preservative.

Plasencia<sup>3</sup> has recently suggested a method which is shorter than Ellinger's and according to its sponsor, just as accurate.

*Calculation.*—Ellinger claims that *one-sixth* of the amount determined must be *added* to the value obtained by titration in order to secure accurate data. This correction should always be made.

#### XIV. Chlorides.

**Dehn-Clark Method.**<sup>4</sup>—In this method the organic compounds, that hold the chlorine too firmly for its quantitative precipitation with silver nitrate, are destroyed by oxidation with sodium peroxide. Sodium peroxide in the presence of water gives off nascent oxygen according to the following equation:



The oxygen then attacks the organic matter and the chlorine is left as sodium chloride. The procedure is as follows: To 10 c.c. of urine

<sup>1</sup> The washing should be continued until the wash water is no longer colored. Ordinarily two or three washings are sufficient. If a separation of indigo particles takes place during the washing process, the wash water should be filtered, the indigo extracted with chloroform, and the usual method applied from this point.

<sup>2</sup> A "stock solution" of potassium permanganate containing 3 grams per liter should be prepared, and when needed for titration purposes a suitable volume of this solution should be diluted with 40 volumes of water. The potassium permanganate solution should be standardized with pure indigo.

<sup>3</sup> Plasencia: *Revista de Medicina y Cirugía.*, 17, 1, 1912.

<sup>4</sup> Private communication to the author from Mr. S. C. Clark.

in a 75-100 c.c. casserole, add 1.0-1.2 gram of sodium peroxide and evaporate the mixture to dryness on a boiling water-bath. In case the residue is not pure white, thus indicating that insufficient sodium peroxide has been added, the residue should be moistened with distilled water, additional sodium peroxide added, and the mixture again evaporated to dryness. When the oxidation is complete, treat the mass with 10-20 c.c. of distilled water and stir until it has practically all been brought into solution. Then introduce a bit of litmus paper and add dilute nitric acid (1:1) until the litmus paper turns red and *all effervescence ceases*. Now place the casserole on a hot plate or on a gauze and heat the contents almost to the boiling-point.<sup>1</sup> To the hot solution add a standard solution of silver nitrate (see page 419) in slight excess.<sup>2</sup> Filter off the silver chloride while the solution is still hot and wash the precipitate thoroughly with distilled water. To the filtrate, add 1 c.c. of a saturated solution of ferric ammonium sulphate and then titrate with a standard solution of ammonium thiocyanate (see page 420) until the clear, slightly yellow fluid (or the opalescent, milky fluid, in case there is much excess of silver nitrate) changes to a slight reddish-brown color. The color of the end-point varies with the individual. The exact end-point reached is not so important as is the securing of the *same* end-point in a series of determinations as that obtained in the standardization of the standard solutions used.

*Calculation.*—The standard solution of silver nitrate should be made up so that 1 c.c. equals 0.010 gram of sodium chloride and 1 c.c. of the ammonium thiocyanate should be equivalent to 1 c.c. of the silver nitrate solution (see p. 419). Then, if the number of cubic centimeters of ammonium thiocyanate used be subtracted from the number of cubic centimeters of silver nitrate, the difference is the number of cubic centimeters of silver nitrate actually used in the precipitation of chlorine as silver chloride. This number, multiplied by 0.010, gives the weight in *grams* of the sodium chloride in the 10 c.c. of urine used. If it is desired to express the result in percentage of sodium chloride, move the decimal point *one* place to the right.

In a similar manner the weight or percentage of *chlorine* may be computed, using the factor 0.006 as explained in Mohr's method, below. Calculate the quantity of sodium chloride and of chlorine in the twenty-four-hour urine specimen.

2. **Mohr's Method.**—To 10 c.c. of urine in a small platinum or porce-

<sup>1</sup> If there is a slight precipitate, due to silicic acid from the casserole, this is filtered off and the filtrate collected in a 200 c.c. beaker.

<sup>2</sup> This point is most easily recognized by keeping the solution hot and in constant agitation while adding the silver nitrate so that the silver chloride formed coagulates and sinks, leaving a clear, supernatant fluid.

lain crucible or dish add about 2 grams of chlorine-free potassium nitrate and evaporate to dryness at  $100^{\circ}$  C. (The evaporation may be conducted over a low flame provided care is taken to prevent loss by spurting.) By means of crucible tongs hold the crucible or dish over a free flame until all carbonaceous matter has disappeared and the fused mass is slightly yellow in color. Cool the residue somewhat and bring it into solution in a small amount (15–25 c.c.) of distilled water acidified with about 10 drops of nitric acid. Transfer the solution to a small beaker, being sure to rinse out the crucible or dish very carefully. Test the reaction of the fluid, and if not already acid in reaction to litmus, render it slightly acid with nitric acid. Now neutralize the solution by the addition of calcium carbonate<sup>1</sup> in substance, add 2–5 drops of neutral potassium chromate solution to the mixture, and titrate with a standard silver nitrate solution.<sup>2</sup>

This standard solution should be run in from a burette, stirring the liquid in the beaker after each addition. The end-reaction is reached when the yellow color of the solution changes to a slight *orange-red*. At this point take the burette reading and compute the percentage of chlorine and sodium chloride in the urine examined.

*Calculation.*—Since 1 c.c. of the standard silver nitrate solution is equivalent to 0.010 gram of sodium chloride, to obtain the *weight*, in grams, of the *sodium chloride* in the 10 c.c. of urine used multiply the number of cubic centimeters of standard solution used by 0.010. If it is desired to express the result in *percentage* of sodium chloride move the decimal point *one place* to the *right*.

To obtain the *weight*, in grams, of the chlorine in the 10 c.c. of urine used multiply the number of cubic centimeters of standard solution used by 0.006, and if it is desired to express the result in *percentage* of chlorine move the decimal point *one place* to the *right*.

Calculate the quantity of sodium chloride and chlorine in the twenty-four-hour urine specimen.

3. **Volhard-Arnold Method.**—Place 10 c.c. of urine in a 100 c.c. volumetric flask, add 20–30 drops of nitric acid (sp. gr. 1.2) and 2 c.c. of a cold saturated solution of ferric alum. If necessary, at this point a few drops of an 8 per cent solution of potassium permanganate may be added to dissipate the red color. Now slowly run in a known volume of the standard silver nitrate<sup>2</sup> solution (20 c.c. is ordinarily used) in order to precipitate the chlorine and insure the presence of an *excess* of silver nitrate. The mixture should be continually shaken during the addition of the

<sup>1</sup> The cessation of effervescence and the presence of some undecomposed calcium carbonate at the bottom of the vessel are the indications of neutralization.

<sup>2</sup> Standard silver nitrate solution may be prepared by dissolving 29.042 grams of silver nitrate in 1 liter of distilled water. Each cubic centimeter of this solution is equivalent to 0.010 gram of sodium chloride or to 0.006 gram of chlorine.

standard solution. Allow the flask to stand 10 minutes, then fill it to the 100 c.c. graduation with distilled water and *thoroughly mix* the contents. Now filter the mixture through a *dry* filter paper, collect 50 c.c. of the filtrate and titrate it with standardized ammonium thiocyanate solution.<sup>1</sup> The first permanent tinge of red-brown indicates the end-point. Take the burette reading and compute the weight of sodium chloride in the 10 c.c. of urine used.

*Calculation.*—The number of cubic centimeters of ammonium thiocyanate solution used indicates the excess of standard silver nitrate solution in the 50 c.c. of filtrate titrated. Multiply this reading by 2, inasmuch as only one-half of the filtrate was employed, and subtract this product from the number of cubic centimeters of silver nitrate (20 c.c.) originally used, in order to obtain the actual number of cubic centimeters of silver nitrate utilized in the precipitation of the chlorides in the 10 c.c. of urine employed.

To obtain the weight in grams of the sodium chloride in the 10 c.c. of urine used, multiply the number of cubic centimeters of the standard silver nitrate solution, actually utilized in the precipitation, by 0.010. If it is desired to express the result in *percentage* of sodium chloride move the decimal point *one* place to the *right*.

In a similar manner the weight, or percentage of *chlorine* may be computed using the factor 0.006 as explained in Mohr's method, page 418.

Calculate the quantity of sodium chloride and chlorine in the twenty-four-hour urine specimen.

4. **Volhard-Harvey Method.**<sup>2</sup>—Introduce 5 c.c. of urine into a small porcelain evaporating dish or casserole and dilute with about 20 c.c. of distilled water. Precipitate the chlorides by the addition of 10 c.c. of standard silver nitrate solution<sup>3</sup> and add 2 c.c. of acidified indicator.<sup>4</sup> Now run in a standard ammonium thiocyanate solution<sup>5</sup> from a burette

<sup>1</sup> This solution is made of such a strength that 1 c.c. of it is equal to 1 c.c. of the standard silver nitrate solution used. To prepare the solution dissolve 13 grams of ammonium thiocyanate,  $\text{NH}_4\text{SCN}$ , in a little less than a liter of water. In a small flask place 20 c.c. of the standard silver nitrate solution, 5 c.c. of the ferric alum solution and 4 c.c. of nitric acid (sp. gr. 1.2), add water to make the total volume 100 c.c. and thoroughly mix the contents of the flask. Now run in the ammonium thiocyanate solution from a burette until a permanent red-brown tinge is produced. This is the end-reaction and indicates that the last trace of silver nitrate has been precipitated. Take the burette reading and calculate the amount of water necessary to use in diluting the ammonium thiocyanate in order that 10 c.c. of this solution may be exactly equal to 10 c.c. of the silver nitrate solution. Make this dilution and titrate again to be certain that the solution is of the proper strength.

<sup>2</sup> Harvey: *Archives of Internal Medicine*, 6, 12, 1910.

<sup>3</sup> See p. 419.

<sup>4</sup> This is prepared as follows: To 30 c.c. of distilled water add 70 c.c. of 33 per cent nitric acid (sp. gr. 1.2) and dissolve 100 grams of crystalline ferric ammonium sulphate in this dilute acid solution. Filter and use the filtrate which is a saturated solution of the iron salt. This single reagent takes the place of the nitric acid and ferric alum as used in Volhard-Arnold method, and insures the use of the proper quantity of acid.

<sup>5</sup> This is a solution of ammonium thiocyanate of such a strength that 2 c.c. is equivalent to 1 c.c. of the silver nitrate solution. First make a concentrated solution by dissolving 13



until a faint red-brown tint is visible throughout the mixture. This point may be determined readily by permitting the precipitate to settle somewhat. Calculate the sodium chloride value so indicated below.

(If a red tint is produced when the first drop of thiocyanate is added an additional 10 c.c. of the standard silver nitrate solution must be introduced. The titration should then proceed as above described and proper allowance made in the calculation for the extra volume of silver nitrate employed.)

*Calculation.*—Since 2 c.c. of the ammonium thiocyanate solution is equivalent to 1 c.c. of the silver nitrate solution, divide the burette reading by 2 and subtract the quotient from 10 c.c., the quantity of silver nitrate solution taken. This value is the number of cubic centimeters of silver nitrate solution actually used in the precipitation of the chlorides. As 1 c.c. of the silver nitrate solution is equivalent to 0.01 gram of sodium chloride, the number of cubic centimeters of silver nitrate solution used multiplied by 0.01 gram will give the weight of *sodium chloride* in the 5 c.c. portion of urine used. The weight of *chlorine* may be computed by using the factor 0.006 as explained under Mohr's method, page 418.

Calculate the weight of sodium chloride and chlorine in the twenty-four-hour urine specimen.

A "short cut" method of calculating the twenty-four-hour output of sodium chloride consists in subtracting the burette reading from 20 c.c., multiplying this value by the total urine volume and pointing off three places.

## XV. Acetone and Diacetic Acid.

1. **Folin-Hart Method.**—This method serves the same purpose as the Messinger-Huppert Method, *i. e.*, the determination of both acetone and diacetic acid in terms of acetone. It is, however, much simpler and less time-consuming. The method includes the transformation of the diacetic acid into acetone and carbon dioxide by means of heat and the subsequent removal of the acetone thus formed, as well as the preformed acetone, by means of an air current as first suggested by Folin (see p. 399). The procedure is as follows: Introduce into a wide-mouthed bottle

grams in one liter of water. To determine the requisite dilution to make such a solution that 2 c.c. shall be equivalent to 1 c.c. of the silver nitrate solution proceed as follows: Introduce 10 c.c. of the silver nitrate solution into a small porcelain evaporating dish or casserole, add 30–50 c.c. of distilled water, 2 c.c. of the acid indicator and titrate as described above with the ammonium thiocyanate solution. The total volume of the concentrated thiocyanate solution including that used in this titration is divided by ten, and the result multiplied by the difference between this burette reading and 20 c.c. This will give the volume of distilled water which must be added to the concentrated thiocyanate solution to render 2 c.c. equivalent to 1 c.c. of the silver nitrate solution.

200 c.c. of water, an accurately measured excess of N/10 iodine solution<sup>1</sup> and an excess of 40 per cent potassium hydroxide. Prepare an aerometer cylinder containing alkaline hypiodite solution to absorb any acetone which may be present in the air of the laboratory, and between the cylinder and bottle suspend a test-tube about two inches in diameter. This large test-tube should contain 20 c.c. of the urine under examination, 10 drops of a 10 per cent solution of phosphoric acid, 10 grams of sodium chloride, and a little petroleum, and should be raised sufficiently high to facilitate the easy application of heat to its bottom portion. The connections on the side of the tube should be provided with bulb-tubes containing cotton. When the apparatus is arranged as described, it should be connected with a Chapman pump and an air current passed through for twenty-five minutes. During this period the contents of the test-tube are heated just to the boiling-point and after an interval of five minutes again heated in the same manner. By this means the diacetic acid is converted into acetone and at the end of the twenty-five-minute period this acetone, as well as the preformed acetone, will have been removed from the urine to the absorption bottle and there retained as iodoform.

The contents of the absorption bottle should now be acidified with concentrated hydrochloric acid,<sup>2</sup> and titrated with N/10 sodium thio-sulphate and starch as in the Messinger-Huppert method (see below).

2. **Messinger-Huppert Method.**<sup>3</sup>—Place 100 c.c. of urine in a distillation flask and add 2 c.c. of 50 per cent acetic acid. Connect the flask with a condenser, properly arrange a receiver, attach a terminal series of bulbs containing water, and distil over about nine-tenths of the urine mixture. Remove the receiver, attach another, and subject the resid-

<sup>1</sup> Proceed as follows in order to obtain a rough idea regarding the amount of N/10 iodine solution to be used: Introduce into a test-tube 10 c.c. of the urine under examination and 1 c.c. of a solution of ferric chloride made by dissolving 100 grams of ferric chloride in 100 c.c. of distilled water. After permitting the mixture to stand for two minutes, compare the color with that of an equal volume of the ferric chloride solution in a test-tube of similar diameter. If the two solutions be of approximately the same color intensity, 20 c.c. of the urine under examination will yield sufficient acetone to require nearly 10 c.c. of N/10 iodine solution. In case the mixture is darker in color than is the ferric chloride solution, the former should be diluted with distilled water until it is of approximately the same intensity as the ferric chloride solution. From this data the amount of N/10 iodine solution required may be roughly estimated by means of the following table:

Urine c.c.	Ferric chloride.	Water.	N/10 Iodine required c.c.
10	1	...	10
10	1	10	20
10	1	20	35
10	1	30	50

<sup>2</sup> An excess of iodine is indicated by the development of a brown color.

<sup>3</sup> This method serves to determine *both* acetone and diacetic acid in *terms of acetone*.

ual portion of the mixture to a second distillation. Test this fluid for acetone and if the presence of acetone is indicated add about 100 c.c. of water to the residue and again distil. Treat the united acetone distillates with 1 c.c. of dilute (12 per cent) sulphuric acid and redistil, collecting this second distillate in a glass-stoppered flask. During distillation, however, the glass stopper is replaced by a cork with a double perforation, the glass tube from one perforation passing to the condenser, while the bulbs containing water, before mentioned, are attached by means of the tube in the other perforation. Allow the distillation process to proceed until practically all of the fluid has passed over, then remove the receiving flask and insert the glass stopper. Now treat the distillate carefully with 10 c.c. of a N/10 solution of iodine and add sodium hydroxide solution, drop by drop, until the blue color is dissipated and the iodoform precipitates. Stopper the flask and shake it for about one minute, acidify the solution with concentrated hydrochloric acid, and note the production of a brown color if an excess of iodine is present. In case there is no such excess, the solution should be treated with N/10 iodine solution until an excess is obtained. Retitrate this excess of iodine with N/10 sodium thiosulphate solution until a light yellow color is observed. At this point a few cubic centimeters of starch paste should be added and the mixture again titrated until no blue color is visible. This is the end-reaction.

*Calculation.*—Subtract the number of cubic centimeters of N/10 thiosulphate solution used from the volume of N/10 iodine solution employed. Since 1 c.c. of the iodine solution is equivalent to 0.967 milligram of acetone, and since 1 c.c. of the thiosulphate solution is equivalent to 1 c.c. of the iodine solution, if we multiply the remainder from the above subtraction by 0.967 we will obtain the number of milligrams of acetone in the 100 c.c. of urine examined.

Calculate the quantity of acetone in the twenty-four-hour urine specimen.

## XVI. Acetone.

1. **Folin's Method.**—The same type of apparatus is used in this method as that described in Folin's method for the determination of ammonia (see p. 399). The procedure is as follows: Introduce 20–25 c.c. of the urine under examination into the aerometer cylinder and add 10 drops of 10 per cent phosphoric acid,<sup>1</sup> 8–10 grams of sodium chloride,<sup>2</sup> and a little petroleum. Introduce into an absorption flask,<sup>3</sup>

<sup>1</sup> Oxalic acid (0.2–0.3 gram) may be substituted if desired.

<sup>2</sup> Acetone is insoluble in a saturated solution of sodium chloride.

<sup>3</sup> Folin's improved absorption tube (see Fig. 128, p. 400) should be used in this connection inasmuch as the original type embracing the use of a rubber stopper is unsatisfactory because of the solvent action of alkaline hypiodite on rubber.

such as is used in the ammonia determination (see p. 399), 150 c.c. of water, 10 c.c. of a 40 per cent solution of potassium hydroxide, and an excess of a N/10 iodine solution. Connect the flask with the aerometer cylinder, attach a Chapman pump, and permit an air current, slightly less rapid than that used for the determination of ammonia, to be drawn through the solution for 20–25 minutes. All of the acetone will, at this point, have been converted into iodoform in the absorption flask. Add 10 c.c. of concentrated hydrochloric acid (a volume equivalent to that of the strong alkali originally added), to the contents of the latter and titrate the excess of iodine by means of N/10 sodium thiosulphate solution and starch, as in the Messinger-Huppert method (see p. 422).

Folin has further made suggestions regarding the *simultaneous* determination of acetone and ammonia by the use of the same air current.<sup>1</sup> This is an important consideration for the clinician inasmuch as urines which contain acetone and diacetic acid are generally those from which the ammonia data are also desired. The procedure for the combination method is as follows: Arrange the ammonia apparatus as usual (see p. 399), and to the aerometer of the ammonia apparatus attach the acetone apparatus set up as described above. Regulate the air current with special reference to the determination of acetone and at the end of 20–25 minutes disconnect the acetone apparatus and complete the determination of the acetone as just described. The air current is not interrupted, and after having run one and one-half hours the ammonia apparatus is detached and the ammonia determination completed as described on page 399.

If data regarding diacetic acid are desired, the result obtained by Folin's method may be subtracted from the result obtained by the Messinger-Huppert method (see p. 422), inasmuch as the latter method determines both acetone and diacetic acid. Under all conditions the determination of acetone should be as expeditious as possible. This is essential, not only because of the fact that any diacetic acid present in the urine will become transformed into acetone, but *also* because of the rapid spontaneous decomposition of the alkaline hypiodite solution used in the determination of the acetone. It has been claimed that alkaline hypiodite solutions are almost completely converted into *iodate* solutions in *one-half hour*. Folin states, however, that the transformation is not so rapid as this, but he nevertheless emphasizes the necessity of rapidity of manipulation. At the same time it should be remembered that the air current must not be as rapid as for ammonia, inasmuch as the alkaline hypiodite solution will not absorb all the acetone under those conditions.

<sup>1</sup> These determinations may even be made on the *same sample* of urine if the sample is too small for the double determination.

## XVII. Diacetic Acid.

1. **Folin-Hart Method.**—Arrange the apparatus as described under the Folin-Hart method for the determination of acetone and diacetic acid (see p. 421). Start the air current in the usual way and permit it to run 25 minutes without the application of heat to the urine under examination. Under these conditions the preformed acetone present in the solution is all removed (see p. 423). Immediately attach a freshly prepared absorption bottle or introduce fresh alkaline hypiodite solution into the original bottle. Apply heat to the large test-tube as already described (see p. 422), in order to convert the diacetic acid into acetone, permit the air current to continue for the usual 25-minute period, and determine the diacetic acid value in terms of acetone by the usual titration procedure (see p. 422).

2. **Folin-Messinger-Huppert Method.**—Determine the combined acetone and diacetic acid, in terms of *acetone*, by the Messinger-Huppert method (see p. 422), and subsequently determine the acetone by Folin's method (see p. 423). Subtract the value determined by the second method from that obtained in the first method to secure data regarding the diacetic acid content of the urine, *in terms of acetone*.

XVIII.  $\beta$ -Oxybutyric Acid.

1. **Shaffer's Method.**—Introduce 25–250 c.c. of urine<sup>1</sup> into a 500 c.c. volumetric flask and add an excess of basic lead acetate and 10 c.c. of concentrated ammonium hydroxide. Dilute the mixture to the 500 c.c. mark, shake the flask thoroughly and filter. Transfer 200 c.c. of the filtrate to an 800 c.c. Kjeldahl distilling flask, add 300–400 c.c. of water, 15 c.c. of concentrated sulphuric acid and a little talcum and distil the mixture until 200 to 250 c.c. of distillate has been collected (A).<sup>2</sup> To this distillate (A), which contains *acetone* (both preformed and that produced from diacetic acid), and *volatile fatty acids* is added 5 c.c. of 10 per cent potassium hydroxide and the distillate redistilled in order to remove the volatile fatty acids.<sup>3</sup> This second distillate (A<sub>2</sub>) is then titrated with standard iodine and thiosulphate (see p. 423). The urine-sulphuric acid residue from which distillate A was obtained is again

<sup>1</sup> The amount used depends upon the expected yield of  $\beta$ -oxybutyric acid. In the case of urines which give a strong ferric chloride reaction for diacetic acid, or when 5–10 grams or more of  $\beta$ -oxybutyric acid is expected, it is unnecessary to use more than 25–50 c.c. of urine. However, in case only a trace of  $\beta$ -oxybutyric acid is expected, the volume should be much larger as indicated. Under all conditions, the amount specified is sufficient for duplicate determinations. It is desirable to use such a volume of urine as contains the proper amount of  $\beta$ -oxybutyric acid to yield 25–50 milligrams of acetone.

<sup>2</sup> This distilling flask should be provided with a dropping tube, by means of which water may be introduced in order to prevent the contents of the flask from becoming less than 400 c.c. in volume. Care should be taken to use a good condenser in the distillation, but it is not necessary to cool the distillate with ice.

<sup>3</sup> Formic acid is one of the most troublesome.

distilled, 400–600 c.c. of a 0.1–0.5 per cent potassium bichromate solution being added, by means of the dropping tube, during the process of distillation.<sup>1</sup> In adding the bichromate, care should be taken not to add it faster than the distillate collects except in cases where the boiling fluid assumes a pure green color, thus indicating that the bichromate is being used up more rapidly. After about 500 c.c. of distillate (B) has collected, 20 c.c. of a 3 per cent solution of hydrogen peroxide and a few cubic centimeters of potassium hydroxide solution are added and the mixture (B) subjected to redistillation. Distil off about 300 c.c. and titrate this distillate (B<sub>2</sub>) as usual with iodine and thiosulphate (see p. 423.)

*Calculation.*—The author advises the use of solutions of thiosulphate and iodine, which are a trifle stronger than N/10; *i. e.*, 103.4 N/10. Each cubic centimeter of an iodine solution of this strength is equivalent to one milligram of acetone or to 1.794 milligrams of  $\beta$ -oxybutyric acid. The thiosulphate solution is accepted as the standard and should be restandardized, from time to time, by a N/10 solution of potassium bi-iodate.

2. **Black's Method.**—Render 50 c.c. of the urine under examination, faintly alkaline with sodium carbonate and evaporate to one-third the original volume. Concentrate to about 10 c.c. on a water-bath, cool the residue, acidify it with a few drops of concentrated hydrochloric acid<sup>2</sup> and add plaster of Paris to form a thick paste. Permit the mixture to stand until it begins to “set,” then break it up with a stout glass rod having a blunt end and reduce the material to the consistency of a fairly dry coarse meal.<sup>3</sup> Transfer the meal to a Soxhlet apparatus and extract with ether for two hours. At the end of this period evaporate the ether-extract either spontaneously or in an air current. Dissolve the residue in water, add a little bone-black, if necessary, filter until a clear solution is obtained and make up the filtrate to a known volume (25 c.c. or less) with water. The  $\beta$ -oxybutyric acid should then be determined by means of the polariscope.

3. **Darmstädter's Method.**—This method is based on the fact that crotonic acid is formed from  $\beta$ -oxybutyric acid under the influence of concentrated mineral acids. The method is as follows: Render 100 c.c. of urine slightly alkaline with sodium carbonate and evaporate nearly to dryness on a water-bath. Dissolve the residue in 150–200 c.c. of 50–55 per cent sulphuric acid, transfer the acid solution to a 1-liter distillation flask and connect it with a condenser. Through the cork of the flask

<sup>1</sup> Generally the addition of 0.5 gram of potassium bichromate is sufficient. In case the urine contains a high concentration of sugar or when a large volume of urine is used, it may be necessary to use 2–3 grams of the bichromate.

<sup>2</sup> The residue should give a distinct red color with litmus paper.

<sup>3</sup> Before this is accomplished it may, in some cases, be necessary to add a little more plaster of Paris.

introduce the stem of a dropping funnel containing water. Heat the flask gently until foaming ceases, then use a full flame and distil over about 300–350 c.c. of fluid, keeping the volume of liquid in the distillation flask constant by the addition of water from the dropping funnel as the distillate collects. Ordinarily it will take about 2–2 1/2 hours to collect this amount of distillate. Extract the distillate three times<sup>1</sup> with ether in a separatory funnel, evaporate the ether and heat the residue at 160° C. for a few minutes to remove volatile fatty acids. Dissolve the residue in 50 c.c. of water, filter and titrate this aqueous solution of crotonic acid with N/10 sodium hydroxide solution, using phenolphthalein as indicator.

*Calculation.*—One c.c. of N/10 sodium hydroxide solution equals 0.0086 gram of crotonic acid, 1 part of crotonic acid equals 1.21 part of  $\beta$ -oxybutyric acid, and 1 c.c. of N/10 sodium hydroxide solution equals 0.01041 gram of  $\beta$ -oxybutyric acid. To compute the quantity of  $\beta$ -oxybutyric acid, in grams, multiply the number of cubic centimeters of N/10 sodium hydroxide solution used by 0.01041.

4. **Bergell's Method.**—Render 100–300 c.c. of sugar-free<sup>2</sup> urine slightly alkaline with sodium carbonate, evaporate the alkaline urine to a syrup on a water-bath, cool the syrup, rub it up with syrupy phosphoric acid (being careful to keep the mixture cool), 20–30 grams of finely pulverized, anhydrous copper sulphate, and 20–25 grams of fine sand. Mix the mass thoroughly, place it in a paper extraction thimble<sup>3</sup> and extract the dry mixture with ether in a Soxhlet apparatus (Fig. 136, page 437). Evaporate the ether, dissolve the residue in about 25 c.c. of water, decolorize the fluid with animal charcoal, if necessary, and determine the content of  $\beta$ -oxybutyric acid by a polarization test.

5. **Boekelman and Bouma's Method.**—Place 25 c.c. of urine in a flask, add 25 c.c. of 12 per cent sodium hydroxide and 25 c.c. of benzoyl chloride, stopper the flask and shake it vigorously for three minutes *under cold water*. Remove the clear fluid by means of a pipette, filter it and subject it to a polarization test. Through the action of the benzoyl chloride all the lævo-rotatory substances except  $\beta$ -oxybutyric acid will have been removed and the lævo-rotation now exhibited by the urine will be due entirely to that acid.

## XIX. Acidity.

**Folin's Method.**—The *total acidity* of urine may be determined as follows: Place 25 c.c. of urine in a 200 c.c. Erlenmeyer flask and add

<sup>1</sup> Shaffer has recently called attention to the fact that it is extremely difficult to extract all of the crotonic acid if but *three* extractions are made

<sup>2</sup> If sugar is present it must be removed by fermentation.

<sup>3</sup> The Schleicher and Schüll fat-free extraction thimble is very satisfactory.

15–20 grams of finely pulverized potassium oxalate and 1–2 drops of a 1 per cent phenolphthalein solution to the fluid. Shake the mixture vigorously for 1–2 minutes and titrate it immediately with N/10 sodium hydroxide until a faint but unmistakable pink remains permanent on further shaking. Take the burette reading and calculate the acidity of the urine under examination.

*Calculation.*—If  $y$  represents the number of cubic centimeters of N/10 sodium hydroxide used and  $y'$  represents the volume of urine excreted in twenty-four hours, the *total acidity* of the twenty-four-hour urine specimen may be calculated by means of the following proportion:

$25:y::y':x$  (acidity of 24-hour urine expressed in cubic centimeters of N/10 sodium hydroxide).

Each cubic centimeter of N/10 sodium hydroxide contains 0.004 gram of sodium hydroxide, and this is equivalent to 0.0063 gram of oxalic acid. Therefore, in order to express the total acidity of the twenty-four-hour urine specimen in equivalent grams of sodium hydroxide, multiply the value of  $x$ , as just determined, by 0.004, or multiply the value of  $x$  by 0.0063 if it is desired to express the total acidity in grams of oxalic acid.

## XX. Purine Bases.

1. **Welker's Modification of the Methods of Arnstein and of Salkowski.**<sup>1</sup>—Four hundred cubic centimeters of urine, free from protein, are treated with 100 c.c. of magnesia mixture and 600 c.c. of water. This is then filtered and of the clear filtrate a measured quantity (600–800 c.c.) is treated with an excess (10 c.c.) of a 3 per cent silver nitrate solution. Concentrated ammonium hydroxide is added in small quantities, with stirring, until all the chlorides have dissolved. Allow the flocculent precipitate of the silver purine compounds to settle to the bottom, then pass the supernatant liquid through the filter before disturbing the precipitate. Finally transfer the precipitate quantitatively to the paper which must be of known nitrogen content. The precipitate is washed with dilute (1 per cent) ammonium hydroxide. The paper with the precipitate is then transferred to a Kjeldahl flask and about 100 c.c. of water and a small quantity (about 0.1 gram) of magnesium oxide are added. The water is then boiled until all the ammonia has been driven off. Test the steam with litmus paper.

The material in the flask is then digested by means of the usual Kjeldahl method (see p. 401). The digestion must be watched care-

<sup>1</sup> Private communication from Dr. W. H. Welker.



fully at the time the sulphuric acid reaches sufficient concentration to affect the filter paper, inasmuch as the  $\text{SO}_2$  produced causes considerable frothing. The *total* nitrogen (purine base, uric acid and filter-paper nitrogen) is now determined in the usual way (see Kjeldahl Method, p. 401). This result *minus* the uric acid and filter-paper nitrogen will give the figure for the purine-base nitrogen.

2. **Krüger and Schmidt's Method.**—This method serves for the determination of both uric acid and the purine bases. The principle involved is the precipitation of both the uric acid and the purine bases in combination with copper oxide and the subsequent decomposition of this precipitate by means of sodium sulphide. The uric acid is then precipitated by means of hydrochloric acid and the purine bases are separated from the filtrate in the form of their copper or silver compounds. The nitrogen content of the precipitates of uric acid and purine bases is then determined by means of the Kjeldahl method (see p. 401) and the corresponding values for uric acid and purine bases calculated. The method is as follows: To 400 c.c. of albumin-free urine<sup>1</sup> in a liter flask,<sup>2</sup> add 24 grams of sodium acetate, 40 c.c. of a solution of sodium bisulphite<sup>3</sup> and heat the mixture to boiling. Add 40–80 c.c.<sup>4</sup> of a 10 per cent solution of copper sulphate and maintain the temperature of the mixture at the boiling-point for at least three minutes. Filter off the flocculent precipitate, wash it with hot water until the wash water is colorless, and return the washed precipitate to the flask by puncturing the tip of the filter paper and washing the precipitate through by means of hot water. Add water until the volume in the flask is approximately 200 c.c., heat the mixture to boiling and decompose the precipitate of copper oxide by the addition of 30 c.c. of sodium sulphide solution.<sup>5</sup> After decomposition is complete, the mixture should be acidified with acetic acid and heated to boiling until the separating sulphur collects in a mass. Filter the hot fluid by means of a filter-pump, wash with *hot* water, add 10 c.c. of 10 per cent hydrochloric acid and evaporate the filtrate in a porcelain dish until the total volume has been reduced to about 10 c.c. Permit this residue to stand about two hours to allow for the separation of the uric acid, leaving the purine

<sup>1</sup> If albumin is present, the urine should be heated to boiling, acidified with acetic acid, and filtered.

<sup>2</sup> The total volume of urine for the twenty-four hours should be sufficiently diluted with water to make the total volume of the solution 1600–2000 c.c.

<sup>3</sup> A solution containing 50 grams of Kahlbaum's commercial sodium bisulphite in 100 c.c. of water.

<sup>4</sup> The exact amount depending upon the content of the purine bases.

<sup>5</sup> This is made by saturating a 1 per cent solution of sodium hydroxide with hydrogen sulphide gas and adding an equal volume of 1 per cent sodium hydroxide.

Ordinarily the addition of 30 c.c. of this solution is sufficient, but the presence of an excess of sulphide should be *proven* by adding a drop of lead acetate to a drop of the solution. Under these conditions a dark brown color will show the presence of an excess of sodium sulphide.

bases in solution. Filter off the precipitate of uric acid, using a small filter paper, and wash the uric acid, with water made acid with sulphuric acid, until the total volume of the original filtrate and the wash water aggregates 75 c.c. Determine the nitrogen content of the precipitate by means of the Kjeldahl method (see p. 401), and calculate the uric acid equivalent.<sup>1</sup>

Render the filtrate from the uric acid crystals alkaline with sodium hydroxide, add acetic acid until faintly acid and heat to 70° C. Now add 1 c.c. of a 10 per cent solution of acetic acid and 10 c.c. of a suspension of manganese dioxide<sup>2</sup> to oxidize the traces of uric acid which remain in the solution. Agitate the mixture for one minute, add 10 c.c. of the sodium bisulphite solution<sup>3</sup> and 5 c.c. of a 10 per cent solution of copper sulphate and heat the mixture to boiling for three minutes. Filter off the precipitate, wash it with *hot* water, and determine its nitrogen content by means of the Kjeldahl method (see p. 401). Inasmuch as the composition and proportion of the purine bases present in urine is variable, no factor can be applied. The result as regards these bases must therefore be expressed in terms of nitrogen.

Benedict and Saiki<sup>4</sup> report cases in which the *total* purine nitrogen by this method was less than the uric-acid nitrogen as determined by the Folin-Shaffer method. The inaccuracy was found to lie in the Krüger and Schmidt method. To obviate this they advise the addition of 20 c.c. of glacial acetic acid for each 300 c.c. of urine employed, the acid being added before the first precipitation.

3. **Salkowski's Method.**—Place 400–600 c.c. of protein-free urine in a beaker. Introduce into another beaker 30–50 c.c. of an ammoniacal silver solution<sup>5</sup> with 30–50 c.c. of magnesia mixture,<sup>6</sup> add some ammonium hydroxide and if necessary some ammonium chloride to clear the solution. Now add this solution to the urine, stirring continually with a glass rod, and allow the mixture to stand for one-half hour. Collect the precipitate on a filter paper, wash it with dilute ammonium hydroxide, and finally wash it back into the original beaker. Suspend the precipitate in 600–800 c.c. of water, add a few drops of hydrochloric acid and decompose it by means of hydrogen sulphide.

<sup>1</sup> This may be done by multiplying the nitrogen value by three and adding three and one-half milligrams to the product as a correction for the uric acid remaining in solution in the 75 c.c.

<sup>2</sup> Made by heating a 0.5 per cent solution of potassium permanganate with a little alcohol until it is decolorized.

<sup>3</sup> To dissolve the excess of manganese dioxide.

<sup>4</sup> Benedict and Saiki: *Jour. Biol. Chem.*, 7, 27, 1909.

<sup>5</sup> Prepared by dissolving 26 grams of silver nitrate in about 500 c.c. of water, adding enough ammonium hydroxide to redissolve the precipitate which forms upon the first addition of the ammonia and making the balance of the mixture up to 1 liter with water.

<sup>6</sup> Directions for preparation may be found on page 313.

Now heat the solution to boiling, filter while hot and evaporate the filtrate to dryness on a water-bath. Extract the residue with 20–30 c.c. of hot 3 per cent sulphuric acid and allow the extract to stand twenty-four hours. Filter off the uric acid, wash it, make the filtrate ammoniacal and precipitate the purine bases again with silver nitrate. Collect this precipitate on a small-sized chlorine-free filter paper, wash, dry, and incinerate it in the usual manner. Now dissolve the ash in nitric acid and titrate with ammonium thiocyanate according to the Volhard-Arnold method (see p. 419). Calculate the content of purine bases in the urine examined, bearing in mind that in an equal mixture of the silver salts of the purine bases, such as we have here, one part of silver corresponds to 0.277 gram of nitrogen or to 0.7381 gram of the bases.

### XXI. Purine Nitrogen.

**Hall's Purinometer.**<sup>1</sup>—By means of the instrument shown in Fig. 133, urine may be examined for total purine nitrogen, *i. e.*, nitrogen in the form of purine bases, urates and uric acid. The method does not give an absolutely accurate measure of the purine values. It is, however, of considerable service clinically. The principle of the method is the preliminary precipitation of the phosphates present followed by the precipitation of the purine bodies in the form of their silver compounds by means of an ammoniacal silver nitrate solution. The volume of this silver precipitate is then determined and its nitrogen value interpolated by means of a table of equivalent values. In using the purinometer proceed as follows: Collect the twenty-four-hour urine and mix it thoroughly. Take 100 c.c. of the urine and if albumin is present make slightly acid with acetic acid and boil and filter. Close the stopcock of the instrument and introduce 90 c.c. of urine and 20 c.c. of a modified magnesia mixture.<sup>2</sup> Turn the stopcock and permit the precipitated phosphates to pass into the lower chamber of the instrument. After an interval of ten minutes has elapsed the stopcock should be closed and suffi-

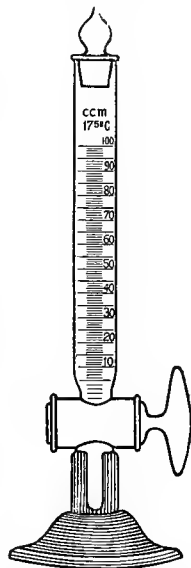


FIG. 133.—HALL'S PURINOMETER.

<sup>1</sup> Hall: *The Purine Bodies*, Philadelphia, 1904.

<sup>2</sup> This is prepared as follows: Dissolve 10 grams of magnesium chloride in about 75 c.c. of water and add 10 grams of ammonium chloride. Introduce 100 c.c. of concentrated ammonium hydroxide into this mixture. If a precipitate forms add ammonium hydroxide until a clear solution is obtained. Make the volume 200 c.c. by means of water and add 10 grams of purified talcum.

cient ammoniacal silver nitrate solution<sup>1</sup> added to make the total volume in the upper chamber 100 c.c. The precipitate of the silver compounds of the purine bodies should be pale yellow. Any silver chloride present may be brought into solution in the strong ammoniacal solution by the repeated inversion of the purinometer. In case the chloride does not dissolve it should be brought into solution by the addition of further ammonium hydroxide. Place the purinometer in a dark room for twenty-four hours and at the end of this time read the volume of the purine precipitate. Interpolate the value in terms of purine nitrogen by means of the following table:

Precipitate c.c.	Purine nitrogen per cent (grams in 100 c.c.)
4	0.0078
5	0.0097
6	0.0117
7	0.0136
8	0.0156
9	0.0175
10	0.0185
11	0.0195
12	0.0205
13	0.0218
14	0.0225
15	0.0234
16	0.0240
17	0.0260
18	0.0265
19	0.0270
20	0.0275
21	0.0283
22	0.0286
23	0.0299
24	0.0312
25	0.0325

*Calculation.*—Multiply the purine nitrogen percentage by the total volume of urine and divide by 100 to obtain the total purine nitrogen value. For example, if the precipitate was found to be 12 c.c. and the total volume of the twenty-four-hour urine was 1300 c.c. the calculation would be as follows:

$$12 \text{ c.c.} = 0.0205 \text{ per cent purine nitrogen.}$$

$$0.0205 \times 13.0 = 0.2665 \text{ gram purine nitrogen.}$$

## XXII. Allantoin.<sup>2</sup>

**Paduschka-Underhill-Kleiner Method.**—To 50–100 c.c. of urine in a beaker add basic lead acetate until no more precipitate forms.

<sup>1</sup> This solution has the following formula:

Silver nitrate	1 gram
Ammonium hydroxide (sp. gr. 0.90)	100 c.c.
Talcum	5 grams
Distilled water	100 c.c.

<sup>2</sup> A much more accurate method has been devised by Wiechowski (*Biochemische Zeitschrift*, 19, 368, 1909.)

Filter and pass hydrogen sulphide gas through an aliquot portion of the filtrate to remove the excess of lead.<sup>1</sup> Filter again, drive off the hydrogen sulphide by heat and treat an aliquot portion of the filtrate with a 10 per cent solution of silver nitrate until precipitation is complete.<sup>2</sup> Filter off this precipitate, wash it with water and determine its nitrogen content by means of the Kjeldahl method (see p. 401). This is the "purine nitrogen." Render an aliquot portion of the filtrate faintly alkaline,<sup>3</sup> with a 1 per cent solution of ammonium hydroxide and add 50–100 c.c. of a 10 per cent solution of silver nitrate. If allantoin be present a white, flocculent precipitate will form and gradually sink to the bottom of the solution. Filter, wash the precipitate free from ammonium hydroxide by means of a 1 per cent solution of sodium sulphate and determine its nitrogen content by the Kjeldahl method (see p. 401).

### XXIII. Oxalic Acid.

**Salkowski-Autenrieth and Barth Method.**—Place the twenty-four-hour urine specimen in a precipitating jar, add an excess of calcium chloride, render the urine strongly ammoniacal, stir it well, and allow it to stand 18–20 hours. Filter off the precipitate, wash it with a small amount of water and dissolve it in about 30 c.c. of a *hot* 15 per cent solution of hydrochloric acid. By means of a separatory funnel extract the solution with 150 c.c. of ether which contains 3 per cent of alcohol, repeating the extraction four or five times with fresh portions of ether. Unite the ethereal extracts, allow them to stand for an hour in a flask, and then filter through a *dry* filter paper. Add 5 c.c. of water to the filtrate, to prevent the formation of diethyl oxalate when the solution is heated, and distil off the ether. If necessary, decolorize the liquid with animal charcoal and filter. Concentrate the filtrate to 3–5 c.c., add a little calcium chloride solution, make it ammoniacal, and after a few minutes render it slightly acid with acetic acid. Allow the acidified solution to stand several hours, collect the precipitate of calcium oxalate on a washed filter paper,<sup>4</sup> wash, incinerate strongly (to CaO), and weigh in the usual manner.

*Calculation.*—Since 56 parts of CaO are equivalent to 90 parts of oxalic acid, the quantity of oxalic acid in the volume of urine taken may be determined by multiplying the weight of CaO by the factor 1.6071.

<sup>1</sup> In the original method of Paduschka sodium sulphate is used for this purpose.

<sup>2</sup> Ordinarily from 20–30 c.c. is required

<sup>3</sup> Using litmus as the indicator.

<sup>4</sup> Schleicher and Schüll, No. 589, is satisfactory.

## XXIV. Total Solids.

1. **Drying Method.**—Place 5 c.c. of urine in a weighed shallow dish, acidify *very slightly* with acetic acid (1–3 drops), and dry it *in vacuo* in the presence of sulphuric acid to constant weight. Calculate the *percentage* of solids in the urine sample and the *total solids* for the twenty-four-hour period.

Practically all the methods the technique of which includes evaporation at an increased temperature, either under atmospheric conditions or *in vacuo*, are attended with error.

Shackell's method<sup>1</sup> which entails the vacuum desiccation of the frozen sample is extremely satisfactory and should be used in all biological work where the greatest accuracy is desired.

2. **Calculation by Long's Coefficient.**—The quantity of solid material contained in the urine excreted for any twenty-four-hour period may be approximately computed by multiplying the second and third decimal figures of the specific gravity by 2.6. This gives us the *number of grams of solid matter in 1 liter of urine*. From this value the total solids for the twenty-four-hour period may easily be determined.

*Calculation.*—If the volume of urine for the twenty-four hours was 1120 c.c. and the specific gravity 1.018, the calculation would be as follows:

$$(a) \quad 16 \times 2.6 = 46.8 \text{ grams of solid matter in 1 liter of urine.}$$

$$(b) \quad \frac{46.8 \times 1120}{1000} = 52.4 \text{ grams of solid matter in 1120 c.c. of urine.}$$

Long's coefficient was determined for urine whose specific gravity was taken at 25° C. and is probably more accurate, for conditions obtaining in America, than the older coefficient of Haeser, 2.33.

<sup>1</sup> Shackell: *American Journal of Physiology*, 24, 325, 1909.

## CHAPTER XXIII.

### QUANTITATIVE ANALYSIS OF MILK, GASTRIC JUICE, AND BLOOD.

#### (a) Quantitative Analysis of Milk.

1. **Specific Gravity.**—This may be determined conveniently by means of a Soxhlet, Veith, or Quevenne *lactometer*. A lactometer reading of  $32^{\circ}$  denotes a specific gravity of 1.032. The determination should be made at about  $60^{\circ}$  F. and the lactometer reading corrected by adding or subtracting  $0.1^{\circ}$  for every degree F. above or below that temperature.

**Fat.** (a) *Babcock's Centrifugal Method.*—The principle of this method is the destruction of organic matter other than fat by sulphuric acid and the centrifugation of the acid solution in the special tube shown in Fig. 134 and the subsequent reading of the percentage of fat by means of the tube's graduated neck. The method is one of the most satisfactory in common use and is accurate to within 0.5 per cent. Proceed as follows: By means of a special narrow pipette introduce milk into the tube up to the 5 c.c. mark. Now add sufficient sulphuric acid (sp. gr. 1.83–1.834) to fill the body of the tube and rotate the tube to secure a homogeneous acid-milk solution. Fill the neck of the tube with an acid-alcohol mixture.<sup>1</sup> Centrifuge the tube and contents for one to two minutes and read off the percentage of fat by means of the graduated neck of the tube. If the top of the fat column is not at zero it may be brought there by the addition of water and a moment's recentrifugation.

In case very rich milk (over 5 per cent fat) is under examination, it may be diluted with an equal volume of water before examination and the fat percentage multiplied by 2. In the examination of cream it is customary to dilute the sample with *four volumes* of water and multiply the resultant fat value by 5.

2. **Fat.**—(b) *Quantitative Determination of Fat in Milk by the Meigs*<sup>2</sup>

<sup>1</sup> This mixture consists of equal volumes of amyl alcohol and concentrated hydrochloric acid.

<sup>2</sup> Original paper by Dr. Arthur V. Meigs in *Philadelphia Medical Times*, July 1, 1882.

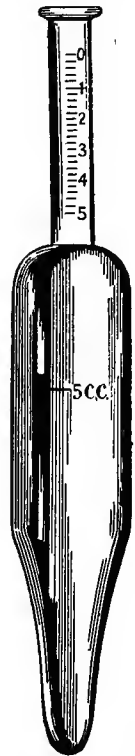


FIG. 134.—BABCOCK TUBE.

*Method with Modification and Improved Apparatus by Croll.*<sup>1</sup>—The method as stated by Dr. Meigs is: Approximately 10 c.c. of milk is carefully weighed and transferred to an ordinary 100 c.c. glass-stoppered graduated cylinder. Twenty c.c. each of distilled water and ether (0.720) are added, the ground-glass stopper tightly inserted in the bottle, and the whole shaken vigorously for five minutes.

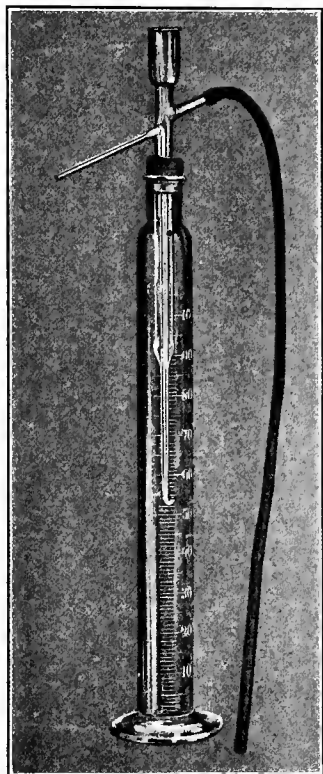


FIG. 135.—CROLL'S FAT APPARATUS.

Then the bottle is carefully unstoppered, 20 c.c. 95 per cent alcohol added, the stopper reinserted and again shaken for five minutes. The bottle is now placed on a table and the contents will separate into two distinct strata, the upper of which contains practically all the fat. This stratum is carefully removed by a small pipette and transferred to a carefully weighed glass evaporating dish. The thin ether layer remaining is washed by the addition of 5 c.c. of ether. This is removed by pipetting off. This washing is repeated four times. On each addition the sides of the bottle should carefully be washed down by the fresh ether. Finally, the pipette is rinsed with a little ether. The evaporating dish with contents is now placed on a safety water-bath and the ether evaporated. The drying is continued in a hot-air oven at a temperature below 100° C. and finally completed in a desiccator to constant weight.

Croll's modification consists of subsequent repeated extraction of the end-product of evaporation with absolute ether. The combined extracts are filtered and the small filter paper is washed repeatedly with absolute ether. The combined extracts and washings are evaporated and dried as before and then weighed.

The piece of apparatus shown in Fig. 135, above was also devised by Croll to do away with the use of the pipette. On closing the top with a finger and blowing into the mouth-piece, the upper stratum is forced out into the dish. The bottle is washed by simply pouring the ether into the tube. This lessens the possibility of accidental loss.

The accuracy of the method compared with that of the Soxhlet method,

<sup>1</sup> Private Communication.



using the paper-coil modification and extracting until fresh portions of absolute ether gave no further trace of extractive material, is shown by the average difference on twelve samples of human milk being only 0.017 per cent less than by the Soxhlet and on seven samples cow's milk being only 0.019 per cent less. The extreme differences in case of the human milk were -0.004 per cent and -0.044 per cent and in case of the cow's milk -0.006 per cent and -0.068 per cent.

(c) *Adams' Paper-coil Method.*—Introduce about 5 c.c. of milk into a small beaker, quickly ascertain the weight to centigrams, stand a fat-free coil<sup>1</sup> in the beaker, and incline the vessel and rotate the coil in order to hasten the absorption of the milk. Immediately upon the complete absorption of the milk remove the coil and again quickly ascertain the weight of the beaker. The difference in the weights of the beaker at the two weighings represents the quantity of milk absorbed by the coil. Dry the coil carefully at a temperature below 100° C. and extract it with ether for 3-5 hours in a Soxhlet apparatus (Fig. 136, p. 437.) Using a safety water-bath, heat the flask containing the fat to constant weight at a temperature below 100° C.

*Calculation.*—Divide the weight of fat, in grams, by the weight of milk, in grams. The quotient is the *percentage of fat* contained in the milk examined.

(d) *Approximate Determination by Feser's Lactoscope.*—Milk is opaque mainly because of the suspended fat globules and therefore by means of the estimation of this opacity we may obtain data as to the *approximate* content of fat. Feser's lactoscope (Fig. 137) may be used for this purpose. Proceed as follows: By means of the graduated pipette accompanying the instrument introduce 4 c.c. of milk into the lactoscope. Add water gradually, shaking after each addition, and note the point at which the black lines upon the inner white glass cylinder are *distinctly* visible. Observe the point on the graduated scale of the lactoscope which is level with the surface of the diluted milk. This reading represents the *per-*

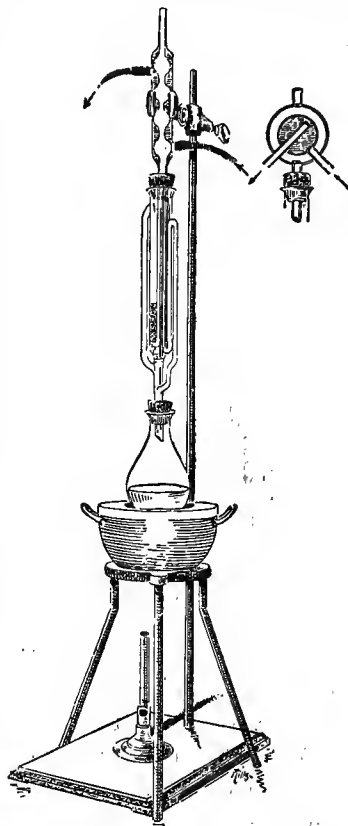


FIG. 136.—SOXHLET APPARATUS.

<sup>1</sup> Very satisfactory coils are manufactured by Schleicher and Schüll.

centage of fat present in the undiluted milk. Pure milk should contain at least 3 per cent of fat.

3. **Total Solids.**<sup>1</sup>—Introduce 2–5 grams of milk into a weighed flat-bottomed platinum dish<sup>2</sup> and quickly ascertain the weight to milligrams. Expel the major portion of the water by heating the open dish on a water-bath and continue the heating in an air-bath or water oven at 97°–100° C. until the weight is constant. (If platinum dishes are employed this residue may be used in the determination of ash according to the method described below.)

*Calculation.*<sup>3</sup>—Divide the weight of the residue, in grams, by the weight of milk used, in grams. The quotient is the percentage of solids contained in the milk examined.



FIG. 137.—FESER'S LACTOSCOPE.

4. **Ash.**—Heat the dry solids from 2–5 grams of milk, obtained according to the method just given, over a very low flame<sup>4</sup> until a white or light gray ash is obtained. Cool the dish in a desiccator and weigh. (This ash may be used in testing for preservatives according to directions on page 244.)

5. **Proteins.**—Introduce a known weight of milk (5–10 grams) into a 500 c.c. Kjeldahl digestion flask and add 20 c.c. of concentrated sulphuric acid and about 0.2 gram of copper sulphate. Expel the major portion of the water by heating over a low flame and finally use a full flame and allow the mixture to boil 1–2 hours. Complete the determination according to the directions given under Kjeldahl Method, page 401.

*Calculation.*—Multiply the total nitrogen content by the factor 6.37<sup>5</sup> to obtain the protein content of the milk examined.

6. **Caseinogen.**—Mix about 20 grams of milk with 40 c.c. of a saturated solution of magnesium sulphate and add the salt in substance until no more will dissolve. The precipitate consists of caseinogen

<sup>1</sup> Shackell's method for the vacuum desiccation of frozen preparations may be used where great accuracy is desired (see *American Journal of Physiology*, 24, 325, 1909).

<sup>2</sup> Lead foil dishes, costing only about one dollar per gross, make a very satisfactory substitute for the platinum dishes.

<sup>3</sup> The percentage of total solids may be calculated from the specific gravity and percentage of fat by means of the following formula which has been proposed by Richmond:

$$S = 0.25 L + 1.2 F + 0.14$$

S = total solids.

L = lactometer reading.

F = fat content.

<sup>4</sup> Great care should be used in this ignition, the dish at no time being heated above a faint redness, as chlorides may volatilize.

<sup>5</sup> The usual factor employed for the calculation of protein from the nitrogen content is 6.25 and is based on the assumption that proteins contain on the average 16 per cent of nitrogen. This special factor of 6.37 is used here to calculate the protein content from the total nitrogen, since the principal protein constituents of milk, *i. e.*, caseinogen and lactalbumin, contain 15.7 per cent. of nitrogen.

admixed with a little fat and lacto-globulin. Filter off the precipitate, wash it thoroughly with a saturated solution of magnesium sulphate,<sup>1</sup> transfer the filter paper and precipitate to a Kjeldahl digestion flask, and determine the nitrogen content according to the directions given in the previous experiment.

*Calculation.*—Multiply the *total nitrogen* by the factor 6.37 to obtain the casein content.

7. **Hart's Caseinogen Method.**<sup>2</sup>—Introduce 10.5 c.c. of milk into a 200 c.c. Erlenmeyer flask and add 75 c.c. of distilled water and 1–1.5 c.c. of 10 per cent acetic acid.<sup>3</sup> Mix the contents by giving the flask a vigorous rotary motion. The precipitated caseinogen is now filtered off upon a 9–11 cm. filter paper.<sup>4</sup> Wash out the adsorbed and loosely combined acetic acid by means of *cold* water. Continue the washing of both the caseinogen on the filter and that adhering to the flask, until the wash water has reached a volume of at least 250 c.c.

Now return the precipitate and paper to the original Erlenmeyer flask, add 75–80 c.c. of neutral (carbon dioxide-free) water, 10 c.c. of N/10 potassium hydroxide and a few drops of phenolphthalein. Stopper the flask and shake it vigorously, by hand or machine, until the caseinogen has been brought into solution.<sup>5</sup> Rinse the stopper with neutral (carbon dioxide-free) water and titrate the alkaline caseinogen solution at once with N/10 hydrochloric acid until there is a disappearance of all red color.<sup>6</sup>

*Calculation.*—Subtract the corrected<sup>5</sup> acid reading from the 10 c.c. of alkali used. The difference is the *percentage of caseinogen in the milk*. For example, if it takes 6.7 c.c. of N/10 hydrochloric acid to titrate the alkaline solution to the end point and the check test was equivalent to 0.2 c.c. N/10 acid the caseinogen value would be obtained as follows:

$$10 - (6.7 + 0.2) = 3.1 \text{ per cent caseinogen.}$$

8. **Lactalbumin.**—To the filtrate and washings from the determi-

<sup>1</sup> Preserve the filtrate and washings for the determination of lactalbumin (Expt. 8).

<sup>2</sup> Hart: *Jour. Biol. Chem.*, 6, 445, 1909.

<sup>3</sup> In general 1.5 c.c. of acetic acid gives a clear solution which filters nicely but occasionally, when the milk has a low caseinogen value it is advisable to use less acetic acid.

<sup>4</sup> The process of filtration may be retarded through the packing of the caseinogen mass upon the filter paper. In this case conduct a fine stream of cold water against the upper point of contact of filter paper and caseinogen. By this means the caseinogen precipitate is loosened and gathers in the apex of the filter. This procedure is very essential. It is not necessary to remove the caseinogen which adheres to the interior of the flask.

<sup>5</sup> Solution is indicated by the disappearance of the white caseinogen particles which would otherwise settle to the bottom of the flask.

<sup>6</sup> A check test should be run parallel with the entire determination. Even with special precautions as to neutrality, it is generally found that an acid check of 0.2–0.3 c.c. will be obtained. This check titration should be *added* to the volume of acid used in titration.

nation of caseinogen, in Experiment 6, add Almén's<sup>1</sup> reagent until no more precipitate forms. Filter off the precipitate and determine the nitrogen content according to the directions given under Proteins, above.

*Calculation.*—Multiply the *total nitrogen* by the factor 6.37 to obtain the lactalbumin content.

8. **Lactose.**—To about 350 c.c. of water in a beaker add 20 grams of milk, mix thoroughly, acidify the fluid with about 2 c.c. of 10 per cent acetic acid and stir the acidified mixture continuously until a flocculent precipitate forms. At this point the reaction should be distinctly acid to litmus. Heat the solution to boiling for one-half hour, filter, rinse the beaker thoroughly, and wash the precipitated proteins and the adherent fat with *hot* water. Combine the filtrate and wash water and concentrate the mixture to about 150 c.c. Cool the solution and dilute it to 200 c.c. in a volumetric flask. Titrate this sugar solution according to directions given under Fehling's Method, page 384.

*Calculation.*—Make the calculation according to directions given under Fehling's Method, p. 384, bearing in mind that 10 c.c. of Fehling's solution is completely reduced by 0.0676 *grams of lactose*.

## (b) Quantitative Analysis of Gastric Juice.

### Töpfer's Method.

This method is much less elaborate than many others but is sufficiently accurate for ordinary clinical purposes. The method embraces the volumetric determination of (1) *total acidity*, (2) *combined acidity*,<sup>2</sup> and (3) *free acidity*, and the subsequent calculation of (4) *acidity due to organic acids and acid salts*, from the data thus obtained.

Strain the gastric contents and introduce 10 c.c. of the strained material into each of three small beakers or porcelain dishes.<sup>3</sup> Label the vessels *A*, *B* and *C*, respectively, and proceed with the analysis according to the directions given below.

1. **Total Acidity.**<sup>4</sup>—Add 3 drops of a 1 per cent alcoholic solution of phenolphthalein<sup>5</sup> to the contents of vessel *A* and titrate with N/10 sodium hydroxide solution until a *faint pink* color is produced which cannot be deepened by further addition of a drop of N/10 sodium hydroxide. Take the burette reading and calculate the total acidity.

*Calculation.*—The total acidity may be expressed in the following ways:

<sup>1</sup> Almén's reagent may be prepared by dissolving 5 grams of tannin in 240 c.c. of 50 per cent alcohol and adding 10 c.c. of 25 per cent acetic acid.

<sup>2</sup> For a discussion of *combined acid* see chapter on Gastric Digestion.

<sup>3</sup> If sufficient gastric juice is not available it may be diluted with water or a smaller amount, e. g., 5 c.c. taken for each determination.

<sup>4</sup> This includes free and combined acid and acid salts.

<sup>5</sup> One gram of phenolphthalein dissolved in 100 c.c. of 95 per cent alcohol.

1. The number of cubic centimeters of N/10 sodium hydroxide solution necessary to neutralize 100 c.c. of gastric juice.

2. The weight (in grams) of sodium hydroxide necessary to neutralize 100 c.c. of gastric juice.

3. The weight (in grams) of hydrochloric acid which the total acidity of 100 c.c. of gastric juice represents, *i. e.*, percentage of hydrochloric acid.

The forms of expression most frequently employed are 1 and 3, preference being given to the former.

In making the calculation note the number of cubic centimeters of N/10 sodium hydroxide required to neutralize 10 c.c. of the gastric juice and multiply it by 10 to obtain the number of cubic centimeters necessary to neutralize 100 c.c. of the fluid. If it is desired to express the acidity of 100 c.c. of gastric juice in terms of hydrochloric acid, by weight, multiply the value just obtained by 0.00365.<sup>1</sup>

2. **Combined Acidity.**<sup>2</sup>—Add 3 drops of sodium alizarin sulphonate solution<sup>3</sup> to the contents of vessel *B* and titrate with N/10 sodium hydroxide solution until a *violet* color is produced. In this titration the red color, which appears after the tinge of yellow due to the addition of the indicator has disappeared, must be entirely replaced by a *distinct violet color*. Take the burette reading and calculate the combined acidity.

*Calculation.*—Since the indicator used reacts to all acidities except combined acidity, in order to determine the number of cubic centimeters of N/10 sodium hydroxide necessary to neutralize the combined acidity of 10 c.c. of the gastric juice, we must subtract the burette reading just obtained from the burette reading obtained in the determination of the total acidity. The data for 100 c.c. of gastric juice may be calculated according to the directions given under Total Acidity, page 440.

3. **Free Acidity.**<sup>4</sup>—Add 4 drops of di-methyl-amino-azobenzene (Töpfer's reagent) solution<sup>5</sup> to the contents of the vessel *C* and titrate with N/10 sodium hydroxide solution until the initial red color is replaced by lemon *yellow*.<sup>6</sup> Take the burette reading and calculate the free acidity.

*Calculation.*—The indicator used reacts only to free acid, hence the number of cubic centimeters of N/10 sodium hydroxide used indicates the volume necessary to neutralize the *free acidity* of 10 c.c. of gastric juice. To determine the data for 100 c.c. of gastric juice proceed according to the directions given under Total Acidity, page 440.

<sup>1</sup> One c.c. of N/10 hydrochloric acid contains 0.00365 gram of hydrochloric acid.

<sup>2</sup> Hydrochloric acid combined with protein material.

<sup>3</sup> One gram of sodium alizarin sulphonate dissolved in 100 c.c. of water.

<sup>4</sup> Hydrochloric acid *not* combined with protein material.

<sup>5</sup> One-half gram dissolved in 100 c.c. of 95 per cent alcohol.

<sup>6</sup> If the lemon yellow color appears as soon as the indicator is added it denotes the *absence* of free acid.

4. **Acidity Due to Organic Acids and Acid Salts.**—This value may be conveniently calculated by subtracting the number of cubic centimeters of  $N/10$  sodium hydroxide used in neutralizing the contents of vessel *C* from the number of cubic centimeters of  $N/10$  sodium hydroxide solution used in neutralizing the contents of vessel *B*. The remainder indicates the number of cubic centimeters of  $N/10$  sodium hydroxide solution necessary to neutralize the acidity due to organic acids and acid salts present in 10 c.c. of gastric juice. The data for 100 c.c. of gastric juice may be calculated according to directions given under Total Acidity, page 440.

### (c) Quantitative Analysis of Blood.

For the methods involved in the quantitative examination of blood see Chapter XII.

## APPENDIX.

**Almen's Reagent.**<sup>1</sup>—Dissolve 5 grams of tannin in 240 c.c. of 50 per cent alcohol and add 10 c.c. of 25 per cent acetic acid.

**Ammoniacal Silver Solution.**<sup>2</sup>—Dissolve 26 grams of silver nitrate in about 500 c.c. of water, add enough ammonium hydroxide to redissolve the precipitate which forms upon the first addition of the ammonium hydroxide and make the volume of the mixture up to 1 liter with water.

**Arnold-Lipliawsky Reagent.**<sup>3</sup>—This reagent consists of two definite solutions which are ordinarily preserved separately and mixed just before using. The two solutions are prepared as follows:

(a) One per cent aqueous solution of potassium nitrite.

(b) One gram of *p*-amino-acetophenon dissolved in 100 c.c. of distilled water and enough hydrochloric acid (about 2 c.c.) added drop by drop, to cause the solution, which is at first yellow, to become entirely colorless. An excess of acid must be avoided.

**Barfoed's Solution.**<sup>4</sup>—Dissolve 4.5 grams of neutral, crystallized copper acetate in 100 c.c. of water and add 1.2 c.c. of 50 per cent acetic acid.

**Baryta Mixture.**<sup>5</sup>—A mixture consisting of one volume of a saturated solution of barium nitrate and two volumes of a saturated solution of barium hydroxide.

**Basic Lead Acetate Solution.**<sup>6</sup>—This solution possesses the following formula:

Lead acetate . . . . .	180 grams.
Lead oxide (Litharge) . . . . .	110 grams.
Distilled water to make . . . . .	1000 grams.

Dissolve the lead acetate in about 700 c.c. of distilled water, with boiling. Add this hot solution to the finely powdered lead oxide and boil for one-half hour with occasional stirring. Cool, filter and add sufficient distilled water to the filtrate to make the weight one kilogram.

**Benedict's Solutions.**<sup>7</sup>—*First Modification.*—Benedict's modified

<sup>1</sup> Ott's precipitation test, p. 339. Determination of lactalbumin, p. 439.

<sup>2</sup> Salkowski's method, page 430.

<sup>3</sup> Arnold-Lipliawsky reaction, page 349.

<sup>4</sup> Barfoed's test, pages 36 and 331.

<sup>5</sup> Isolation of urea from urine, page 287.

<sup>6</sup> Indican determination, page 416.

<sup>7</sup> Benedict's modifications of Fehling's test, pages 328 and 329, and Benedict's Method No. 1, page 385.

Fehling solution consists of two definite solutions — a copper sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

*Copper sulphate solution* = 34.65 grams of copper sulphate dissolved in water and made up to 500 c.c.

*Alkaline tartrate solution* = 100 grams of anhydrous sodium carbonate and 173 grams of Rochelle salt dissolved in water and made up to 100 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

*Second Modification.*—Very recently Benedict has further modified his solution and has succeeded in obtaining one which does not deteriorate upon long standing. It has the following composition:

Copper sulphate.....	17.3 grams.
Sodium citrate.....	173.0 grams.
Sodium carbonate.....	100.0 grams.
Distilled water to make 1 liter.	

With the aid of heat dissolve the sodium citrate and carbonate in about 600 c.c. of water. Pour (through a folded filter paper if necessary) into a glass graduate and make up to 850 c.c. Dissolve the copper sulphate in about 100 c.c. of water and make up to 150 c.c. Pour the carbonate-citrate solution into a large beaker or casserole and add the copper sulphate solution slowly, with constant stirring. The mixed solution is ready for use and does not deteriorate upon long standing.

Benedict's solution as used in the *quantitative determination* of sugar (Method No. 1) consists of *three* separate solutions, the two mentioned under *First Modification* and in addition a *potassium ferro-thiocyanate solution*. This third solution contains 15 grams of potassium ferrocyanide, 62.5 grams of potassium thiocyanate and 50 grams of anhydrous sodium carbonate dissolved in water and made up to 500 c.c. In preparing the Benedict's solution for *quantitative* work the *three* solutions mentioned are combined in equal parts.

#### **Benedict's Sugar Reagent (Method No. 2).<sup>1</sup>**

Copper sulphate (crystallized).....	18.0 grams.
Sodium carbonate (crystallized, one-half the weight of the anhydrous salt may be used).....	200.0 grams.
Sodium or potassium citrate.....	200.0 grams.
Potassium thiocyanate.....	125.0 grams.
Potassium ferrocyanide (5 per cent solution).....	5.0 c.c.
Distilled water to make a total volume of.....	1000.0 c.c.

With the aid of heat dissolve the carbonate, citrate and thiocyanate in enough water to make about 800 c.c. of the mixture and filter if necessary.

<sup>1</sup> Quantitative determination of sugar, page 385.



Dissolve the copper sulphate separately in about 100 c.c. of water and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanide solution, cool and dilute to exactly 1 liter. Of the various constituents, the copper salt only need be weighed with exactness. Twenty-five cubic centimeters of the reagent are reduced by 50 mg. of glucose.

### Bial's Reagent.<sup>1</sup>

Orcinol.....	1.5 grams.
Fuming HCl.....	500.00 grams.
Ferric chloride (10 per cent).....	20-30 drops.

### Benedict's Sulphur Reagent.

Crystallized copper nitrate, sulphur-free or of known sulphur content.....	200 grams.
Sodium or potassium chlorate.....	50 grams.
Distilled water to.....	1000 c.c.

**Black's Reagent.**<sup>2</sup>—Made by dissolving 5 grams of ferric chloride and 0.4 gram of ferrous chloride in 100 c.c. of water.

**Blood Serum.**—This may easily be obtained in quantity by the procedure described under Hemagglutination in the chapter on Blood.

**Boas' Reagent.**<sup>3</sup>—Dissolve 5 grams of resorcinol and 3 grams of sucrose in 100 c.c. of 50 per cent alcohol.

**Bonnano's Reagent.**—Dissolve 2 grams of sodium nitrite in 100 c.c. of concentrated hydrochloric acid.

**Bottu's Reagent.**—To 3.5 grams of *o*-nitrophenylpropionic acid add 5 c.c. of a freshly prepared 10 per cent solution of sodium hydroxide and make the volume of the solution one liter with distilled water.

**Combined Hydrochloric Acid (Protein Salt).**—To prepare so-called *combined* hydrochloric acid simply add a soluble protein such as Witte's peptone to *free* hydrochloric acid of the desired strength until it no longer responds to free acid tests (see chapter on Gastric Digestion). For example, if 0.2 per cent combined acid is required the protein would be added to 0.2 per cent *free* hydrochloric acid.

Strictly speaking there is no such thing as "combined" acid in this sense. When the protein is added a *protein salt* of the acid is formed which ionizes differently from the free acid.

**Congo Red.**<sup>4</sup>—Dissolve 0.5 gram of congo red in 90 c.c. of water and add 10 c.c. of 95 per cent alcohol.

**Cross and Bevan's Reagent.**—Combine *two* parts of concentrated hydrochloric acid and *one* part of zinc chloride *by weight*.

<sup>1</sup> Test for pentose, page 352.

<sup>2</sup> Black's reaction, page 350.

<sup>3</sup> Test for free acid, page 130.

<sup>4</sup> Test for free acid, page 130.

**Ehrlich's Diazo Reagent.**<sup>1</sup>—Two separate solutions should be prepared and mixed in definite proportions when needed for use.

(a) Five grams of sodium nitrite dissolved in 1 liter of distilled water.

(b) Five grams of sulphanilic acid and 50 c.c. of hydrochloric acid in 1 liter of distilled water.

Solutions *a* and *b* should be preserved in well-stoppered vessels and mixed in the proportion 1:50 when required. Green asserts that greater delicacy is secured by mixing the solutions in the proportion 1:100. The sodium nitrite deteriorates upon standing and becomes unfit for use in the course of a few weeks.

**Esbach's Reagent.**<sup>2</sup>—Dissolve 10 grams of picric acid and 20 grams of citric acid in 1 liter of water.

**Fehling's Solution.**<sup>3</sup>—Fehling's solution is composed of two definite solutions—a copper sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

*Copper sulphate solution* = 34.65 grams of copper sulphate dissolved in water and made up to 500 c.c.

*Alkaline tartrate solution* = 125 grams of potassium hydroxide and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

**Ferric Alum Solution.**<sup>4</sup>—A cold saturated solution.

**Folin-Shaffer Reagent.**<sup>5</sup>—This reagent consists of 500 grams of ammonium sulphate, 5 grams of uranium acetate, and 60 c.c. of 10 per cent acetic acid in 650 c.c. of distilled water.

**Furfurol Solution.**<sup>6</sup>—Add 1 c.c. of furfurol to 1000 c.c. of distilled water.

**Gallic Acid Solution.**<sup>7</sup>—A saturated alcoholic solution.

**Gies' Biuret Reagent.**—This reagent consists of 10 per cent KOH solution to which enough 3 per cent  $\text{CuSO}_4$  solution has been added to impart a slight though distinct blue color to the clear liquid. The  $\text{CuSO}_4$  should be added drop by drop with thorough shaking after each addition.

**Guaic Solution.**<sup>8</sup>—Dissolve 0.5 gram of guaiac resin in 30 c.c. of 95 per cent alcohol.

<sup>1</sup> Ehrlich's diazo reaction, page 359.

<sup>2</sup> Esbach's method, page 383.

<sup>3</sup> Fehling's method, page 384. Fehling's test, pages 32 and 327.

<sup>4</sup> Volhard-Arnold method, page 419.

<sup>5</sup> Folin-Shaffer method, page 389.

<sup>6</sup> Mylius's modification of Pettenkofer's test, pages 164 and 344. v. Udránsky's test, pages 164 and 344.

<sup>7</sup> Gallic acid test, page 243.

<sup>8</sup> Guaic test, pages 186, 209 and 240.

**Günzberg's Reagent.**<sup>1</sup>—Dissolve 2 grams of phloroglucinol and 1 gram of vanillin in 100 c.c. of 95 per cent alcohol.

**Hammarsten's Reagent.**<sup>2</sup>—Mix 1 volume of 25 per cent nitric acid and 19 volumes of 25 per cent hydrochloric acid and add 1 volume of this acid mixture to 4 volumes of 95 per cent alcohol. It is preferable that the acid mixture be prepared in advance and allowed to stand until yellow in color before adding it to the alcohol.

**Hayem's Solution.**—This solution has the following formula:

Mercuric chloride.....	0.25 grams.
Sodium chloride.....	0.5 grams.
Sodium sulphate.....	2.5 grams.
Distilled water.....	100.0 grams.

**Hopkins-Cole Reagent.**<sup>3</sup>—To one liter of a saturated solution of oxalic acid add 60 grams of sodium amalgam and allow the mixture to stand until the evolution of gas ceases. Filter the dilute with 2-3 volumes of water.

**Hopkins-Cole Reagent (Benedict's Modification).**—Ten grams of powdered magnesium are placed in a large Erlenmeyer flask and shaken up with enough distilled water to liberally cover the magnesium. Two hundred and fifty cubic centimeters of a cold, saturated solution of oxalic acid is now added slowly. The reaction proceeds very rapidly and with the liberation of much heat, so that the flask should be cooled under running water during the addition of the acid. The contents of the flask are shaken after the addition of the last portion of the acid and then poured upon a filter, to remove the insoluble magnesium oxalate. A little wash water is poured through the filter, the filtrate acidified with acetic acid to prevent the partial precipitation of the magnesium on long standing, and made up to a liter with distilled water. This solution contains only the magnesium salt of glyoxylic acid.

**Hypobromite Solution.**<sup>4</sup>—The ingredients of this solution should be prepared in the form of *two* separate solutions which may be united as needed.

(a) Dissolve 125 grams of sodium bromide in water, add 125 grams of bromine and make the total volume of the solution 1 liter.

(b) A solution of sodium hydroxide having a specific gravity of 1.25. This is approximately a 22.5 per cent solution.

Preserve both solutions in rubber-stoppered bottles and when needed for use mix one volume of solution *a*, one volume of solution *b*, and 3 volumes of water.

<sup>1</sup> Test for free acid, page 130.

<sup>2</sup> Hammarsten's reaction, pages 163 and 343.

<sup>3</sup> Hopkins-Cole reaction, page 98.

<sup>4</sup> Methods for determination of urea, page 392.

**Iodine Solution.**<sup>1</sup>—Prepare a 2 per cent solution of potassium iodide and add sufficient iodine to color it a deep yellow.

**Iodine-Zinc Chloride Reagent.**<sup>2</sup>—Dissolve 20 grams of zinc chloride in 8.5 c.c. of water. Cool, and introduce iodine solution (3 grams KI + 1.5 gram I in 60 c.c. of water) drop by drop until iodine begins to precipitate.

**Jolles' Reagent.**<sup>3</sup>—This reagent has the following composition:

Succinic acid.....	40 grams.
Mercuric chloride.....	20 grams.
Sodium chloride.....	20 grams.
Distilled water.....	1000 grams.

**Kantor and Gies' Biuret Paper.**<sup>4</sup>—Immerse filter paper in Gies' Biuret Reagent (p. 99) then dry and cut into strips.

**Kraut's Reagent.**<sup>5</sup>—Dissolve 272 grams of potassium iodide in water and add 80 grams of bismuth subnitrate dissolved in 200 grams of nitric acid (sp. gr. 1.18). Permit the potassium nitrate to crystallize out, then filter it off and make the filtrate up to 1 liter with water.

**Lugol's Solution.**<sup>6</sup>—Dissolve 4 grams of iodine and 6 grams of potassium iodide in 100 c.c. of distilled water.

**Magnesia Mixture.**<sup>7</sup>—Dissolve 175 grams of magnesium sulphate and 350 grams of ammonium chloride in 1400 c.c. of distilled water. Add 700 grams of concentrated ammonium hydroxide, mix thoroughly, and preserve the mixture in a glass-stoppered bottle.

**Millon's Reagent.**<sup>8</sup>—Digest 1 part (by weight) of mercury with 2 parts (by weight) of nitric acid (sp. gr. 1.42) and dilute the resulting solution with 2 volumes of water.

**Molisch's Reagent.**<sup>9</sup>—A 15 per cent alcoholic solution of  $\alpha$ -naphthol.

**Molybdic Solution.**<sup>10</sup>—Molybdic solution is prepared as follows, the parts being *by weight*

Molybdic acid.....	1 part.
Ammonium hydroxide (sp. gr. 0.96).....	4 parts.
Nitric acid (sp. gr. 1.2).....	15 parts.

**Moreigne's Reagent.**<sup>11</sup>—Combine 20 grams of sodium tungstate,

<sup>1</sup> Iodine test, page 50.

<sup>2</sup> Amyloid formation, p. 54.

<sup>3</sup> Jolles' reaction, pages 105 and 334.

<sup>4</sup> Protein tests, p. 332.

<sup>5</sup> Rosenheim's bismuth test for choline, page 273.

<sup>6</sup> Gunning's iodoform test, page 346, and Bardach's reaction, page 101.

<sup>7</sup> Sodium hydroxide and potassium nitrate fusion method for determination of total phosphorus, page 414.

<sup>8</sup> Millon's reaction, page 97.

<sup>9</sup> Molisch's reaction, page 27.

<sup>10</sup> Sodium hydroxide and potassium nitrate fusion method for determination of total phosphorus, page 414.

<sup>11</sup> Moreigne's reaction, page 293.

10 grams of phosphoric acid (sp. gr. 1.13) and 100 c.c. of water. Boil the mixture for twenty minutes, add water to make the volume of the solution equivalent to the original volume, and acidify with hydrochloric acid.

**Mörner's Reagent.**<sup>1</sup>—Thoroughly mix 1 volume of formalin, 45 volumes of distilled water, and 55 volumes of concentrated sulphuric acid.

**Nakayama's Reagent.**<sup>2</sup>—Prepared by combining 99 c.c. of alcohol and 1 c.c. of fuming hydrochloric acid containing 4 grams of ferric chloride per liter.

#### Nessler-Winkler Solution.

Mercuric iodide.....	10 grams.
Potassium iodide.....	5 grams.
Sodium hydroxide.....	20 grams.
Water.....	100 c.c.

The mercuric iodide is rubbed up in a small porcelain mortar with water, then washed into a flask and the potassium iodide added. The sodium hydroxide is dissolved in the remaining water and the cooled solution added to the above mixture. The solution cleared by standing is preserved in a dark bottle.

**Neutral Olive Oil.**<sup>3</sup>—Shake ordinary olive oil with a 10 per cent solution of sodium carbonate, extract the mixture with ether, and remove the ether by evaporation. The residue is *neutral* olive oil.

**Nylander's Reagent.**<sup>4</sup>—Digest 2 grams of bismuth subnitrate and 4 grams of Rochelle salt in 100 c.c. of a 10 per cent solution of potassium hydroxide. The reagent should then be cooled and filtered.

**Obermayer's Reagent.**<sup>5</sup>—Add 2-4 grams of ferric chloride to a liter of hydrochloric acid (sp. gr. 1.19).

**Oxalated Plasma.**<sup>6</sup>—Allow arterial blood to run into an equal volume of 0.2 per cent ammonium oxalate solution.

**Para-dimethylaminobenzaldehyde Solution.**<sup>7</sup>—This solution is made by dissolving 5 grams of para-dimethylaminobenzaldehyde in 100 c.c. of 10 per cent sulphuric acid.

**Para-phenylenediamine Hydrochloride Solution.**<sup>8</sup>—Two grams dissolved in 100 c.c. of water.

**Phenolphthalein.**<sup>9</sup>—Dissolve 1 gram of phenolphthalein in 100 c.c. of 95 per cent alcohol.

<sup>1</sup> Mörner's test, page 91.

<sup>2</sup> Nakayama's reaction, pages 162 and 342.

<sup>3</sup> Emulsification of fats, page 143.

<sup>4</sup> Nylander's test, pages 34 and 330.

<sup>5</sup> Obermayer's test, page 299.

<sup>6</sup> Experiments on blood plasma, page 214.

<sup>7</sup> Herter's para-dimethylaminobenzaldehyde reaction, page 176.

<sup>8</sup> Detection of hydrogen peroxide, page 244.

<sup>9</sup> Töpfer's method, page 440.

**Phenylhydrazine Mixture.**<sup>1</sup>—This mixture is prepared by combining 1 part of phenylhydrazine-hydrochloride and 2 parts of sodium acetate *by weight*. These are thoroughly mixed in a mortar.

**Phenylhydrazine-acetate Solution.**<sup>2</sup>—This solution is prepared by mixing 1 volume of glacial acetic acid, 1 volume of water, and 2 volumes of phenylhydrazine (the base).

**Purdy's Solution.**<sup>3</sup>—Purdy's solution has the following composition:

Copper sulphate .....	4.75 <sup>2</sup>	grams.
Potassium hydroxide .....	23.5	grams.
Ammonia (U. S. P., sp. gr. 0.9) .....	350.0	c.c.
Glycerol .....	38.0	c.c.
Distilled water, to make total volume 1 liter.		

**Roberts' Reagent.**<sup>4</sup>—Mix 1 volume of concentrated nitric acid and 5 volumes of a saturated solution of magnesium sulphate.

**Rosenheim's Iodo-Potassium Iodide Solution.**<sup>5</sup>—Dissolve 2 grams of iodine and 6 grams of potassium iodide in 100 c.c. of water.

**Salted Plasma.**<sup>6</sup>—Allow arterial blood to run into an equal volume of a saturated solution of sodium sulphate or a 10 per cent solution of sodium chloride. Keep the mixture in the cold room for about 24 hours.

**Schiff's Reagent.**<sup>7</sup>—This reagent consists of a mixture of three volumes of concentrated sulphuric acid and one volume of 10 per cent ferric chloride.

**Schweitzer's Reagent.**<sup>8</sup>—Add potassium hydroxide to a solution of copper sulphate which contains some ammonium chloride. Filter off the precipitate of cupric hydroxide, wash it, and bring 3 grams of the moist cupric hydroxide into solution in a liter of 20 per cent ammonium hydroxide.

**Seliwanoff's Reagent.**<sup>9</sup>—Dissolve 0.05 gram of resorcinol in 100 c.c. of dilute (1:2) hydrochloric acid.

**Sherrington's Solution.**<sup>10</sup>—This solution possesses the following formula:

Methylene-blue.....	0.1	gram.
Sodium chloride.....	1.2	gram.
Neutral potassium oxalate.....	1.2	gram.
Distilled water.....	300.0	grams.

**Sodium Acetate Solution.**<sup>11</sup>—Dissolve 100 grams of sodium acetate

<sup>1</sup> Phenylhydrazine reaction, pages 28 and 324.

<sup>2</sup> Phenylhydrazine reaction, pages 28 and 324.

<sup>3</sup> Purdy's method, page 387.

<sup>4</sup> Roberts' ring test, pages 104 and 334.

<sup>5</sup> Rosenheim's periodide test, page 273.

<sup>6</sup> Experiments on blood plasma, page 214.

<sup>7</sup> Schiff's reaction, pages 166 and 272.

<sup>8</sup> Schweitzer's solubility test, page 54.

<sup>9</sup> Seliwanoff's reaction, pages 40 and 356.

<sup>10</sup> "Blood counting," page 224.

<sup>11</sup> Uranium acetate method, page 413.

in 800 c.c. of distilled water, add 100 c.c. of 30 per cent acetic acid to the solution, and make the volume of the mixture up to 1 liter with distilled water.

**Sodium Alizarin Sulphonate.**<sup>1</sup>—Dissolve 1 gram of sodium alizarin sulphonate in 100 c.c. of water.

**Sodium Sulphide Solution.**<sup>2</sup>—Saturate a 1 per cent solution of sodium hydroxide with hydrogen sulphide gas and add an equal volume of 1 per cent sodium hydroxide.

**Solera's Test Paper.**<sup>3</sup>—Saturate a good quality of filter paper with 0.5 per cent starch paste to which has been added sufficient iodic acid to make a 1 per cent solution of iodic acid and allow the paper to dry in the air. Cut it in strips of suitable size and preserve for use.

**Spiegler's Reagent.**<sup>4</sup>—This reagent has the following composition:

Tartaric acid.....	20 grams.
Mercuric chloride.....	40 grams.
Glycerol.....	100 grams.
Distilled water.....	1000 grams.

**Standard Ammonium Thiocyanate Solution.**<sup>5</sup>—This solution is made of such a strength that 1 c.c. of it is equal to 1 c.c. of the standard silver nitrate solution mentioned below. To prepare the solution dissolve 12.9 grams of ammonium thiocyanate,  $\text{NH}_4\text{SCN}$ , in a little less than a liter of water. In a small flask place 20 c.c. of the standard silver nitrate solution, 5 c.c. of a cold saturated solution of ferric alum and 4 c.c. of nitric acid (sp. gr. 1.2), add water to make the total volume 100 c.c., and thoroughly mix the contents of the flask. Now run in the ammonium thiocyanate solution from a burette until a permanent *red-brown* tinge is produced. This is the end-reaction and indicates that the last trace of silver nitrate has been precipitated. Take the burette reading and calculate the amount of water necessary to use in diluting the ammonium thiocyanate in order that 10 c.c. of this solution may be exactly equal to 10 c.c. of the silver nitrate solution. Make the dilution and titrate again to be certain that the solution is of the proper strength.

**Standard Silver Nitrate Solution.**<sup>6</sup>—Dissolve 29.042 grams of silver nitrate in 1 liter of distilled water. Each cubic centimeter of this solution is equivalent to 0.01 gram of sodium chloride or to 0.006 gram of chlorine.

**Standard Uranium Acetate Solution.**<sup>7</sup>—Dissolve about 34 grams of

<sup>1</sup> Töpfer's method, page 440.

<sup>2</sup> Krüger and Schmidt's method, pages 391 and 429.

<sup>3</sup> Solera's reaction, page 64.

<sup>4</sup> Spiegler's ring test, pages 104 and 334.

<sup>5</sup> Volhard-Arnold method, page 419, and Dehn-Clark method, page 417.

<sup>6</sup> Volhard-Arnold method, page 419, Mohr's method, page 418, and Dehn-Clark method, page 417.

<sup>7</sup> Uranium acetate method, page 413.

uranium acetate in 1 liter of water. One c.c. of such a solution should now be made equivalent to 0.005 gram of  $P_2O_5$ , phosphoric anhydride. It may be standardized as follows: To 50 c.c. of a standard solution of disodium hydrogen phosphate, of such a strength that the 50 c.c. contains 0.1 gram of  $P_2O_5$ , add 5 c.c. of the sodium acetate solution mentioned on p. 450 and titrate with the uranium solution to the correct end-reaction as indicated in the method proper on p. 413. Inasmuch as 1 c.c. of the uranium solution should precipitate 0.005 gram of  $P_2O_5$ , exactly 20 c.c. of the uranium solution should be required to precipitate the 50 c.c. of the standard phosphate solution. If the two solutions do not bear this relation to each other they must be brought into the proper relation by diluting the uranium solution with distilled water or by increasing its strength.

**Starch Iodide Solution.**<sup>1</sup>—Mix 0.1 gram of starch powder with cold water in a mortar and pour the suspended starch granules into 75–100 c.c. of boiling water, stirring continuously. Cool the starch paste, add 20–25 grams of potassium iodide and dilute the mixture to 50 c.c. This solution deteriorates upon standing, and therefore must be freshly prepared as needed.

**Starch Paste.**<sup>2</sup>—Grind 2 grams of starch powder in a mortar with a small amount of water. Bring 200 c.c. of water to the boiling-point and add the starch mixture from the mortar with continuous stirring. Bring again to the boiling-point and allow it to cool. This makes an approximate 1 per cent starch paste which is a very satisfactory strength for general use.

**Stokes' Reagent.**<sup>3</sup>—A solution containing 2 per cent ferrous sulphate and 3 per cent tartaric acid. When needed for use a small amount should be placed in a test-tube and ammonium hydroxide added until the precipitate which forms on the first addition of the hydroxide has entirely dissolved. This produces *ammonium ferrotartrate* which is a reducing agent.

**Suspension of Manganese Dioxide.**<sup>4</sup>—Made by heating a 0.5 per cent solution of potassium permanganate with a little alcohol until it is decolorized.

**Tanret's Reagent.**<sup>5</sup>—Dissolve 1.35 grams of mercuric chloride in 25 c.c. of water, add to this solution 3.32 grams of potassium iodide dissolved in 25 c.c. of water, then make the total solution up to 60 c.c. with distilled water and add 20 c.c. of glacial acetic acid to the mixture.

<sup>1</sup> Fehling's method, page 384.

<sup>2</sup> Fehling's method, page 384.

<sup>3</sup> Hæmoglobin, page 216. Hæmochromogen, page 219.

<sup>4</sup> Krüger and Schmidt's method, pages 391 and 429.

<sup>5</sup> Tanret's test, pages 104 and 334.



**Tincture of Iodine.**<sup>1</sup>—Dissolve 70 grams of iodine and 50 grams of potassium iodide in 1 liter of 95 per cent alcohol.

**Toison's Solution.**<sup>2</sup>—This solution has the following formula:

Methyl violet.....	0.025	gram.
Sodium chloride.....	1.0	gram.
Sodium sulphate.....	8.0	grams.
Glycerol.....	30.0	grams.
Distilled water.....	160.0	grams.

**Töpfer's Reagent.**<sup>3</sup>—Dissolve 0.5 gram of di-methylaminoazobenzene in 100 c.c. of 95 per cent alcohol.

**Tropæolin OO.**<sup>4</sup>—Dissolve 0.05 gram of tropæolin OO in 100 c.c. of 50 per cent alcohol.

**Uffelmann's Reagent.**<sup>5</sup>—Add a 5 per cent solution of ferric chloride to a 1 per cent solution of carbolic acid until an amethyst-blue color is obtained.

<sup>1</sup> Smith's test, pages 163 and 343.

<sup>2</sup> "Blood counting," page 224.

<sup>3</sup> Töpfer's method, page 440.

<sup>4</sup> Test for free acid, page 130.

<sup>5</sup> Uffelmann's reaction, page 136.



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